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I, LEANNE MYNOTT, MANAGER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003906286 for a patent by GARVAN INSTITUTE OF MEDICAL RESEARCH as filed on 14 November 2003.

WITNESS my hand this
Twenty-fifth day of November 2004

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PROVISIONAL SPECIFICATION

Invention Title:

Novel method of modulating fat deposition and metabolism and metabolic rate

The invention is described in the following statement:

Methods of treatment of feeding disorders and disorders of glucose uptake or metabolism and identifying therapeutic reagents therefor

Field of the invention

The present invention relates generally to the treatment of feeding disorders and disorders of glucose uptake or metabolism, such as, for example, diabetes, obesity, anorexia or bulimia, in humans and other animals. More particularly, this invention provides methods of modifying adipose tissue (e.g., in connection with treating diabetes, obesity, or hypolipidemia) and/or feeding behavior (e.g in connection with treating overeating, bulimia or anorexia). The invention also relates to method for identifying modulators of glucose uptake or metabolism that are useful in the therapeutic methods described herein, e.g. using a non-human animal model.

Background to the invention

1. General

This specification contains nucleotide and amino acid sequence information prepared using PatentIn Version 3.1. Each nucleotide sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, <213> etc). The length and type of sequence (DNA, protein (PRT), etc), and source organism for each nucleotide sequence, are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide sequences referred to in the specification are defined by the term "SEQ ID NO:", followed by the sequence identifier (eg. SEQ ID NO: 1 refers to the sequence in the sequence listing designated as <400>1).

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide other than Thymine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

As used herein the term "derived from" shall be taken to indicate that a specified integer may be obtained from a particular source albeit not necessarily directly from that source.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but not the exclusion of any other step or element or integer or group of elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

The present invention is performed without undue experimentation using, unless otherwise indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, peptide synthesis in solution, solid phase peptide synthesis, and immunology. Such procedures are described, for example, in the following texts that are incorporated by reference:

1. Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Second Edition (1989), whole of Vols I, II, and III;
2. DNA Cloning: A Practical Approach, Vols. I and II (D. N. Glover, ed., 1985), IRL Press, Oxford, whole of text;

3. Oligonucleotide Synthesis: A Practical Approach (M. J. Gait, ed., 1984) IRL Press, Oxford, whole of text, and particularly the papers therein by Gait, pp1-22; Atkinson *et al.*, pp35-81; Sproat *et al.*, pp 83-115; and Wu *et al.*, pp 135-151;

4. Nucleic Acid Hybridization: A Practical Approach (B. D. Hames & S. J. Higgins, eds., 1985) IRL Press, Oxford, whole of text;

5. Animal Cell Culture: Practical Approach, Third Edition (John R.W. Masters, ed., 2000), ISBN 0199637970, whole of text;

6. Immobilized Cells and Enzymes: A Practical Approach (1986) IRL Press, Oxford, whole of text;

7. Perbal, B., A Practical Guide to Molecular Cloning (1984);

8. Methods In Enzymology (S. Colowick and N. Kaplan, eds., Academic Press, Inc.), whole of series;

9. J.F. Ramalho Ortigão, "The Chemistry of Peptide Synthesis" *In:* Knowledge database of Access to Virtual Laboratory website (Interactiva, Germany);

10. Sakakibara, D., Teichman, J., Lien, E. Land Fenichel, R.L. (1976). *Biochem. Biophys. Res. Commun.* **73** 336-342

11. Merrifield, R.B. (1963). *J. Am. Chem. Soc.* 85, 2149-2154.

12. Barany, G. and Merrifield, R.B. (1979) in *The Peptides* (Gross, E. and Meienhofer, J. eds.), vol. 2, pp. 1-284, Academic Press, New York.

13. Wünsch, E., ed. (1974) *Synthese von Peptiden in Houben-Weyls Metoden der Organischen Chemie* (Müler, E., ed.), vol. 15, 4th edn., Parts 1 and 2, Thieme, Stuttgart.

14. Bodanszky, M. (1984) *Principles of Peptide Synthesis*, Springer-Verlag, Heidelberg.

15. Bodanszky, M. & Bodanszky, A. (1984) *The Practice of Peptide Synthesis, Springer-Verlag*, Heidelberg.

16. Bodanszky, M. (1985) *Int. J. Peptide Protein Res.* **25**, 449-474.

17. Handbook of Experimental Immunology, Vols. I-IV (D. M. Weir and C. C. Blackwell, eds., 1986, Blackwell Scientific Publications).

2. Description of the related art

The c-Cbl protein is a multi-adaptor protein that is involved in ligand-induced down regulation of receptor tyrosine kinases. The interaction between c-Cbl and its many

binding partners involves particular c-Cbl protein domains. All Cbl proteins have a high degree of sequence homology between their tyrosine kinase-binding, linker and RING finger domains, and most have extensive proline-rich regions in their carboxy-terminal halves. The tyrosine kinase-binding domain is composed of three interacting domains comprising a four-helix bundle, a Ca^{2+}-binding EF hand, and an atypical or variant Src homology region 2 (SH2) domain that is connected to the RING finger by a short linker domain. A ubiquitin-associated (UBA)/LZ domain at the carboxyl terminus of c-Cbl, Cbl-b and D-Cbl has homology to known ubiquitin-associated domains and leucine zippers and is believed to be involved in Cbl-mediated ubiquitination of active receptors, which is essential for receptor degradation and turnover, thereby leading to cessation of downstream signalling from the receptor (Soubeyran *et al., Nature 416*, 183-187, 2002).

Diabetes, and conditions related thereto such as obesity, are major health concerns throughout the world, and contribute to morbidity and mortality. Non-insulin dependent diabetes mellitus (NIDDM or type II diabetes), is the major form of diabetes in developed countries, however efficient means of therapeutic intervention are lacking. While a large number of environmental and genetic factors contribute to the risk of NIDDM in the United States, prolonged obesity is by far the largest risk factor.

Before the development of diabetes, many obese patients develop a peripheral resistance to the actions of insulin. The molecular basis of insulin-resistance in obesity has been the subject of intensive study, but nonetheless remains elusive. Insights into components and mechanisms of the link between obesity and insulin resistance have been gained from mouse models of obesity which display obesity-induced insulin resistance. The molecular basis of the various mouse obesity models covers a range of mechanisms; nonetheless these all develop diabetes, either before or after the onset of obesity.

Obesity in humans and rodents is also commonly associated with insulin resistance in fat and muscle cells (LeRoith et al., Diabetes Mellitus: a Fundamental and Clinical Text. (Lippincott-Raven, Philadelphia, 1996); DeFronzo et al., Diabetes Care 15:318-68 (1992); Rifkin et al., Diabetes Mellitus, (Elsevier, N.Y., 1990)).

Much work has focussed on the insulin-sensitive glucose transporter GLUT4 (Watson and Pessin, Exp. Cell Res. 271: 75-83, 2001; Bogan et al. published U.S. Patent Application No. 20020052012). Insulin binds to the insulin receptor (IR) in the plasma membrane, where it activates tyrosine kinase in a cascade of events involving phosphatidylinositol 3-kinase (PI 3-K)-mediated recruitment of GLUT4 to the cell surface Min et al., Mol. Cell. 3: 751-760, 1999; Olson et al., Mol. Cell. Biol. 17: 2425-2435, 1997; Hausdorff et al., J. Biol. Chem. 270: 12965-12968, 1995; Elmendorf et al., J. Biol. Chem. 273: 13289-13296, 1998; Holman et al., J. Biol. Chem. 265: 18172-18179, 1990; Piper et al., Am. J. Physiol. 260: C570-C580). The activation of PI 3-K However, PI-K3-mediated trafficking of GLUT4 is not sufficient to explain the extent of insulin resistance (Pessin et al., J. Clin. Invest. 106: 165-169, 2000). For example, other growth factors and adhesion molecules that can activate PI 3-K and its downstream kinases (ie. AKT, protein kinase Cζ/λ (PKCζ/λ)) have no effect on glucose transport or GLUT4 translocation. Additionally, mutant mice lacking GLUT4 develop only mild hyperinsulinemia (Katz et al., Nature 377:151-155, 1995).

A second PI 3K-independent signalling cascade, initiated by recruitment of c-Cbl (Langdon et al., J. Virol. 63: 5420-5424, 1989; Langdon et al., Proc. Natl Acad. Sci. USA 86: 1168-1172, 1989) to the insulin receptor, has been postulated to be involved in insulin-stimulated glucose transport and uptake in fat and muscle cells. In this pathway, the c-Cbl protein is also recruited to the insulin receptor by interaction with the adaptor protein CAP, through one of three SH3 domains in the carboxyl-terminus of CAP. c-Cbl is then phosphorylated by the receptor, and the CAP-Cbl complex dissociates from the insulin receptor and moves to a caveolin-enriches triton insoluble membrane fraction. Based upon two-hybrid assay data measuring *in vitro* protein-protein associations Baumann et al., Nature 407: 202-207, 2000, showed that c-Cbl forms a ternary complex with two other proteins, CAP and flotillin. The interaction with flotillin directs the CAP-Cbl complex to the lipid raft sub-domain of the plasma membrane. Baumann et al. Nature 407: 202-207, 2000, also showed that both insulin-stimulated glucose transport and GLUT4 translocation to the cell surface are attenuated by about 50% in 3T3-L1 adipocytes by expression of a truncated CAP protein lacking SH3 domains (i.e. CAPΔSH3). If this pathway were to operate in fat cells and/or muscle cells *in vivo*, it would be expected that insulin-induced glucose uptake and its subsequent incorporation into both glycogen and lipid would be

impaired in situations which disrupt formation or activity of the c-Cbl-CAP-flotillin complex.

An interaction between c-Cbl and APS, an adaptor protein having a PH domain and SH2 domain (Ahmed et al., Biochem. J. 341, 665-668, 1999) is required for c-Cbl to bind to the insulin receptor in the caveolae-small invaginations in the plasma membrane that are a subset of the lipid raft domains. In this pathway, APS interacts with the phosphorylated insulin receptor via its SH2 domain, and subsequently undergoes tyrosine phosphorylation at a specific residue in the C-terminus of the protein. Upon phosphorylation, APS recruits c-Cbl to the receptor through an atypical SH2 domain of c-Cbl (Saltiel and Pessin, TRENDS Cell Biol. 12, 65-71, 2002).

Based upon co-localization studies, Chiang et al., Nature 410: 944-948, 2001 also showed that the time-course for the insulin-stimulated migration of c-Cbl parallels movement of the SH2-containing adaptor protein CrkII, and the guanyl nucleotide exchange factor C3G, into the caveolin-enriches triton insoluble membrane fraction. Chiang et al. also demonstrated that C3G exchanges GTP for GDP on TC10, a Rho-family GTP-binding protein that regulates GLUT4 transport. In this pathway, phosphorylated c-Cbl recruits CrkII to the lipid rafts, along with C3G to facilitate activation of TC10 by C3G (Chiang et al., Nature 410: 944-948, 2001).

No direct role for c-Cbl in modulating glucose uptake or GLUT4 translocation has been demonstrated in muscle and fat cells *in vivo*. This is because the binding studies by Baumann et al. and Chiang et al. *supra* were carried out in isolated 3T3L1 adipocytes and do not suggest that equivalent effects occur *in vivo*, let alone in *both* muscle and fat cells of animals. It is also unclear whether the effects reported by Baumann et al. for the over expression of CAPΔSH3 protein in isolated adipocytes were a direct consequence of CAP failing to bind c-Cbl or to a secondary effect of expressing the mutant CAPΔSH3 protein. For example, the expressed CAPΔSH3 protein may have modified the ability of endogenous CAP to bind other proteins in the lipid rafts or elsewhere in the cell. Over expression of the CAPΔSH3 protein also decreases insulin-stimulated recruitment of C3G into lipid rafts, and reduces the basal level of activated TC10 in 3T3-Li adipocytes (Chiang et al., Nature 410: 944-948, 2001). Accordingly, no clear direct role has emerged for c-Cbl in modulating glucose uptake

and incorporation into lipid in both fat cells and muscle cells of animals. Thus, it is not possible at present to conclude that impaired c-Cbl-CAP-flotillin complex formation and activity are sufficient to produce insulin resistance in humans and other animals.

A number of mouse models have been developed having genetic obesity-diabetes syndromes (Herberg, et al., Metabolism 26: 59-99, 1977). The mice typically have hyperglycemia, hyperinsulinemia, and obesity, albeit to different degrees, with different times of onset, and for different reasons. In the yellow obese mouse ($A^{y/a}$), a dominant mutation causes the ectopic, ubiquitous expression of the agouti protein, resulting in a condition similar to adult-onset obesity and non-insulin-dependent diabetes mellitus (Michaud et al., Proc Natl Acad Sci USA 91: 2562-2566, 1994). Obese (ob/ob) (Zhang et al., Nature 372: 425-432, 1994); diabetes (db/db) (Tartaglia et al., Cell 83: 1263-1271, 1995), adipose (cpe/cpe) (Naggert et al., Nat. Genet. 10: 135-142, 1995) and tubby (tub/tub) (Kleyn et al., Cell 85: 281-290, 1996; Noben-Trauth et al., Nature 380: 534-548, 1996) are mutations in the genes encoding leptin, the leptin receptor, carboxypeptidase E, and a member of a new family of genes encoding tubby-like proteins, respectively. Obese mice exhibit hyperglycemia, glucose intolerance, and elevated plasma insulin, which develops after the onset of obesity. In db/db mice, elevation of plasma insulin occurs at 2 weeks of age, preceding the onset of obesity at 3-4 weeks and elevation of blood glucose levels at 4-8 weeks. Adipose mice have hyperinsulinemia throughout life in association with hypertrophy and hyperplasia of the islets of Langerhans, with transient hyperglycemia. Tubby mice have normal blood glucose, however plasma insulin is elevated prior to obvious signs of obesity, and the islets of Langerhans are enlarged.

Mouse models having impaired glucose uptake are highly desirable. By virtue of examining their phenotype, such models would have utility in determining appropriate targets for the therapy of a wide range of disorders associated with aberrant glucose metabolism and for determining the efficacy or specificity of therapeutics.

Summary of the invention

In work leading up to the present invention, the inventors studied the role of Cbl, particularly c-Cbl, in insulin resistance, obesity and diabetes *in vivo*. Surprisingly, the inventors found that a targeted disruption of a Cbl locus is mice sufficient to prevent

functional Cbl protein from being expressed, does not disrupt, has little or no defect on basal or insulin-induced glucose uptake and incorporation of glucose into lipid in adipocytes. In muscle cells, Cbl-deficient mice were found to have elevated basal glucose uptake, consistent with an enhanced metabolic rate. Cbl-deficient mice had significantly smaller adipocytes than wild-type counterparts of the same approximate weight. Consistent with this conclusion, the Cbl-deficient mice also had an elevated temperature relative to normal mice, suggesting that the enhanced ratio of lean muscle mass to body fat in Cbl-deficient mice is a consequence of enhanced metabolic rate. Furthermore, acetyl-CoA carboxylase (ACC) phosphorylation was increased in Cbl-deficient mice. Phosphorylation of ACC is associated with reduced ACC activity resulting in increased fatty acid oxidation and ATP synthesis. It was also suprising that the feeding behaviour of the animals was markedly modified, as Cbl-deficient mice exhibited a markedly enhanced appetite as determined by the amount of food consumed per day (dietary intake). These data also indicate that Cbl, such as, for example, c-Cbl, is directly involved in modulating the feeding behaviour of animals and in regulating fat deposition in adipocytes in mammals. The Cbl protein and Cbl-deficient mouse model are therefore useful for identifying compounds that specifically modulate a Cbl-mediated characteristic such as, for example, feeding behaviour (e.g. in the treatment of anorexia or bulimia), fat deposition, metabolic rate, the ratio of lean muscle mass to body fat, or glucose uptake (e.g. in the treatment of obesity or type II diabetes). These modulators are identified by screening animals for their effect on the phenotype of the mouse model, or alternatively, by directly sceening the Cbl protein for altered function, including but not limited to any specific known functions of Cbl such as, for example, binding activity, ubiquitin ligase activity, or by screening for the effect of the compound on the extent of tyrosine phosphorylation of Cbl.

Accordingly, one aspect of the present invention provides a method of identifying a compound that suppresses or reduces feeding behaviour, such as, for example, in the treatment of obesity, said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (b) determining the feeding behaviour of the animal, wherein reduced appetite or dietary intake of the animal compared to the appetite or dietary intake of a Cbl-deficient animal to which the compound has not

been administered indicates that the compound suppresses or reduces feeding behaviour.

In an alternative embodiment, the present invention provides a method for identifying a compound that suppresses or reduces feeding behaviour, such as, for example, in the treatment of obesity, said method comprising determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein enhanced ubiquitin ligase activity of the Cbl protein in the presence of the compound indicates that the compound suppresses or reduces feeding behaviour. In an alternative embodiment, the present invention provides a method for identifying a compound that supresses or reduces feeding behaviour comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein enhanced phosphorylation of tyrosine residues in the Cbl protein in the presence of the compound indicates that the compound suppresses or reduces feeding behaviour.

Another embodiment of the invention provides a method of identifying a compound that enhances feeding behaviour, such as, for example, in the treatment of anorexia or bulimia, said method comprising: (a) administering a compound that suppresses appetite or dietary intake to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal and determining the feeding behaviour of the animal; (b) administering a compound to the animal and determining the feeding behaviour of the animal, wherein enhanced appetite or dietary intake at (b) compared to (a) indicates that the compound enhances feeding behaviour.

In an alternative embodiment, the invention provides a method of identifying a compound that enhances feeding comprising determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein reduced ubiquitin ligase activity of the Cbl protein in the presence of the compound indicates that the compound enhances feeding behaviour.

In an alternative embodiment, the present invention provides a method for identifying a compound that enhances feeding behaviour comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein reduced phosphorylation of tyrosine residues in the Cbl protein in the presence of the compound indicates that the compound enhances feeding behaviour.

In an alternative embodiment, the invention provides a method of identifying a compound that modulates feeding behaviour, such as, for example, in the treatment of anorexia or bulimia, said method comprising: (a) administering a compound to a non-human animal expressing a functional Cbl protein and determining the feeding behaviour of the animal; (b) determining the feeding behaviour of a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (c) comparing the feeding behaviour of the animals at (a) and (b) wherein a comparable feeding behaviour between (a) and (b) indicates that the compound modulates feeding behaviour.

In an alternative embodiment, the invention provides a method of identifying a compound that modulates feeding comprising determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein modified ubiquitin ligase activity of the Cbl protein in the presence of the compound indicates that the compound enhances feeding behaviour.

In an alternative embodiment, the present invention provides a method for identifying a compound that modulates feeding behaviour comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein modified phosphorylation of tyrosine residues in the Cbl protein in the presence of the compound indicates that the compound modulates feeding behaviour.

A further aspect of the present invention provides a method of identifying a compound that enhances fat deposition or reduces lean muscle mass or enhances the ratio of body fat to muscle or reduces metabolic rate such as, for example, in the treatment of

hypolipidemia (eg. as observed in subjects suffering from abetalipoproteinemia, malnutrition or hematologic malignancies, such as acute myelocytic leukemia or chronic myelocytic leukemia), said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (b) determining the fat content of the animal, wherein enhanced fat content of the animal compared to the fat content of a Cbl-deficient animal to which the compound has not been administered indicates that the compound enhances fat deposition or reduces lean muscle mass or enhances the ratio of body fat to muscle or reduces metabolic rate.

In an alternative embodiment, the present invention provides a method of identifying a compound that enhances fat deposition or reduces lean muscle mass or enhances the ratio of body fat to muscle or reduces metabolic rate comprising determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein enhanced ubiquitin ligase activity of the Cbl protein in the presence of the compound indicates that the compound enhances fat deposition or reduces lean muscle mass or enhances the ratio of body fat to muscle or reduces metabolic rate.

In an alternative embodiment, the present invention provides a method for identifying a compound that enhances fat deposition or reduces lean muscle mass or enhances the ratio of body fat to muscle or reduces metabolic rate comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein enhanced phosphorylation of tyrosine residues in the Cbl protein in the presence of the compound indicates that the compound enhances fat deposition or reduces lean muscle mass or enhances the ratio of body fat to muscle or reduces metabolic rate .

In an alternative embodiment, the invention provides a method of identifying a compound that reduces fat deposition or enhances lean muscle mass or reduces the ratio of body fat to muscle or enhances metabolic rate, such as, for example, in the treatment of obesity or neurodegenerative disorders or for cosmetic purposes such as bodybuilding or weight loss, said method comprising: (a) administering a compound that enhances fat deposition or glucose uptake to a genetically modified non-human

animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal and determining the fat content of the animal; (b) administering a compound to the animal and determining the fat content of the animal, wherein a similar or reduced fat content at (b) compared to (a) indicates that the compound reduces fat deposition or enhances lean muscle mass or reduces the ratio of body fat to muscle or enhances metabolic rate.

In an alternative embodiment, the invention provides a method of identifying a compound that reduces fat deposition or enhances lean muscle mass or reduces the ratio of body fat to muscle or enhances metabolic rate comprising: (a) administering a compound to a non-human animal expressing a functional Cbl protein and determining the fat content of the animal; (b) determining the fat content of a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (c) comparing the fat contents of the animals at (a) and (b) wherein a comparable fat content between (a) and (b) indicates that the compound reduces fat deposition or enhances lean muscle mass or reduces the ratio of body fat to muscle or enhances metabolic rate.

In an alternative embodiment, the present invention provides a method of identifying a compound that reduces fat deposition or enhances lean muscle mass or reduces the ratio of body fat to muscle or enhances metabolic rate comprising determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein reduced ubiquitin ligase activity of the Cbl protein in the presence of the compound indicates that the compound reduces fat deposition or enhances lean muscle mass or reduces the ratio of body fat to muscle or enhances metabolic rate.

In an alternative embodiment, the present invention provides a method for identifying a compound that reduces fat deposition or enhances lean muscle mass or reduces the ratio of body fat to muscle or enhances metabolic rate comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein reduced phosphorylation of tyrosine residues in the Cbl protein in the presence of the compound indicates that the compound reduces fat deposition or

enhances lean muscle mass or reduces the ratio of body fat to muscle or enhances metabolic rate .

A further aspect of the present invention provides a method of identifying a compound that reduces the amount of a phosphorylated acetyl CoA carboxylase (ACC) enzyme and/or enhances the activity of an ACC enzyme in a cell, such as, for example, in the treatment of hypolipidemia (eg. as observed in subjects suffering from abetalipoproteinemia, malnutrition or hematologic malignancies, such as acute myelocytic leukemia or chronic myelocytic leukemia), said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (b) determining the activity and/or amount of phosphorylated ACC enzyme in the animal, wherein a reduced amount of phosphorylated ACC enzyme and/or enhanced activity of an ACC enzyme compared to the amount of phosphorylated ACC enzyme or activity of an ACC enzyme of a Cbl-deficient animal to which the compound has not been administered indicates that the compound reduces the amount of a phosphorylated acetyl CoA carboxylase (ACC) enzyme or enhances the activity of an ACC enzyme in a cell.

In an alternative embodiment, the present invention provides a method of identifying a compound that reduces the amount of a phosphorylated acetyl CoA carboxylase (ACC) enzyme and/or enhances the activity of an ACC enzyme in a cell comprising determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein enhanced ubiquitin ligase activity of the Cbl protein in the presence of the compound indicates that the compound reduces the amount of a phosphorylated acetyl CoA carboxylase (ACC) enzyme and/or enhances the activity of an ACC enzyme in a cell.

In an alternative embodiment, the present invention provides a method of identifying a compound that reduces the amount of a phosphorylated acetyl CoA carboxylase (ACC) enzyme and/or enhances the activity of an ACC enzyme in a cell comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein enhanced phosphorylation of tyrosine residues in

the Cbl protein in the presence of the compound reduces the amount of a phosphorylated acetyl CoA carboxylase (ACC) enzyme and/or enhances the activity of an ACC enzyme in a cell.

In an alternative embodiment, the invention provides a method of identifying a compound that enhances the amount of a phosphorylated acetyl CoA carboxylase (ACC) enzyme and/or reduces the activity of an ACC enzyme in a cell , such as, for example, in the treatment of obesity or neurodegenerative disorders or for cosmetic purposes such as bodybuilding or weight loss, said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic mutation within an allele of its Cbl locus that reduces or prevents expression of functional Cbl in said animal; and (b) determining the activity and/or amount of phosphorylated ACC enzyme in the animal, wherein an enhanced level of phosphorylated enzyme and/or reduced activity of an ACC enzyme compared to the level of phosphorylated enzyme and/or activity of an ACC enzyme of a Cbl-deficient animal to which the compound has not been administered indicates that the compound enhances the amount of phosphorylated ACC enzyme and/or reduces the activity of an ACC enzyme.

In an alternative embodiment, the invention provides a method of identifying a compound that enhances the amount of a phosphorylated ACC enzyme and/or reduces the activity of an ACC enzyme comprising: (a) administering a compound to a non-human animal expressing a functional Cbl protein and determining the activity and/or amount of phosphorylated ACC enzyme; (b) determining the activity and/or amount of phosphorylated ACC enzyme in a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (c) comparing the activity and/or amount of phosphorylated ACC enzyme in the animals at (a) and (b) wherein a comparable activity and/or amount of phosphorylated ACC enzyme between (a) and (b) indicates that the compound enhances the amount of a phosphorylated ACC enzyme and/or reduces the activity of an ACC enzyme.

In an alternative embodiment, the present invention provides a method of identifying a compound that enhances the amount of a phosphorylated ACC enzyme and/or

reduces the activity of an ACC enzyme determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein reduced ubiquitin ligase activity of the Cbl protein in the presence of the compound indicates that the compound enhances the amount of a phosphorylated ACC enzyme and/or reduces the activity of an ACC enzyme.

In an alternative embodiment, the present invention provides a method for identifying a compound that enhances the amount of a phosphorylated ACC enzyme and/or reduces the activity of an ACC enzyme comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein reduced phosphorylation of tyrosine residues in the Cbl protein in the presence of the compound indicates that the compound enhances the amount of a phosphorylated ACC enzyme and/or reduces the activity of an ACC enzyme.

A further aspect of the present invention provides a method of identifying a compound that reduces fatty acid oxidation and/or enhances fatty acid synthesis, such as, for example, in the treatment of hypolipidemia (eg. as observed in subjects suffering from abetalipoproteinemia, malnutrition or hematologic malignancies, such as acute myelocytic leukemia or chronic myelocytic leukemia), said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (b) determining the amount of fatty acid synthesis and/or fatty acid oxidation in the animal or a cell derived from the animal, wherein a reduced amount of fatty acid oxidation and/or a reduced amount of fatty acid synthesis compared to the amount of acid oxidation and/or amount of fatty acid synthesis of a Cbl-deficient animal to which the compound has not been administered indicates that the compound reduces fatty acid oxidation and/or enhances fatty acid synthesis.

In an alternative embodiment, the present invention provides a method of identifying a compound that reduces fatty acid oxidation and/or enhances fatty acid synthesis comprising determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein enhanced ubiquitin ligase activity of the Cbl protein

in the presence of the compound indicates that the compound reduces fatty acid oxidation and/or enhances fatty acid synthesis.

In an alternative embodiment, the present invention provides a method of identifying a compound that reduces fatty acid oxidation and/or enhances fatty acid synthesis comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein enhanced phosphorylation of tyrosine residues in the Cbl protein in the presence of the compound reduces fatty acid oxidation and/or enhances fatty acid synthesis.

In an alternative embodiment, the invention provides a method of identifying a compound that enhances fatty acid oxidation and/or reduces fatty acid synthesis, such as, for example, in the treatment of obesity or neurodegenerative disorders or for cosmetic purposes such as bodybuilding or weight loss, said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic mutation within an allele of its Cbl locus that reduces or prevents expression of functional Cbl in said animal; and (b) determining the amount of fatty acid synthesis and/or fatty acid oxidation in the animal or a cell derived from the animal, wherein an enhanced level of fatty acid oxidation and/or reduced level of fatty acid synthesis compared to the level of fatty acid synthesis and/or fatty acid oxidation of a Cbl-deficient animal to which the compound has not been administered indicates that the compound enhances fatty acid oxidation and/or reduces fatty acid synthesis.

In an alternative embodiment, the invention provides a method of identifying a compound that enhances fatty acid oxidation and/or reduces fatty acid synthesis comprising: (a) administering a compound to a non-human animal expressing a functional Cbl protein and determining the amount of fatty acid synthesis and/or fatty acid oxidation in the animal or a cell derived from the animal; (b) determining the amount of fatty acid synthesis and/or fatty acid oxidation in a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal or a cell derived therefrom; and (c) comparing the amount of fatty acid synthesis and/or fatty acid oxidation in the animals or cells at (a) and (b) wherein a comparable amount of fatty acid synthesis and/or fatty acid oxidation between (a) and

(b) indicates that the compound enhances fatty acid oxidation and/or reduces fatty acid synthesis.

In an alternative embodiment, the present invention provides a method of identifying a compound that enhances fatty acid oxidation and/or reduces fatty acid synthesis comprising determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein reduced ubiquitin ligase activity of the Cbl protein in the presence of the compound indicates that the compound enhances fatty acid oxidation and/or reduces fatty acid synthesis .

In an alternative embodiment, the present invention provides a method for identifying a compound that enhances fatty acid oxidation and/or reduces fatty acid synthesis comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein reduced phosphorylation of tyrosine residues in the Cbl protein in the presence of the compound indicates that the compound enhances fatty acid oxidation and/or reduces fatty acid synthesis.

A further aspect of the present invention provides a method of identifying a compound that reduces ATP synthesis, said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (b) determining the amount of ATP synthesis in the animal or a cell derived from the animal, wherein a reduced amount of fatty ATP synthesis compared to the amount of ATP synthesis of a Cbl-deficient animal to which the compound has not been administered indicates that the compound reduces ATP synthesis.

In an alternative embodiment, the present invention provides a method of identifying a compound that reduces ATP synthesis comprising determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein enhanced ubiquitin ligase activity of the Cbl protein in the presence of the compound indicates that the compound reduces ATP synthesis.

In an alternative embodiment, the present invention provides a method of identifying a compound that reduces ATP synthesis comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein enhanced phosphorylation of tyrosine residues in the Cbl protein in the presence of the compound reduces fatty ATP synthesis.

In an alternative embodiment, the invention provides a method of identifying a compound that enhances ATP synthesis, such as, for example, for cosmetic purposes such as bodybuilding, said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic mutation within an allele of its Cbl locus that reduces or prevents expression of functional Cbl in said animal; and (b) determining the amount of ATP synthesis in the animal or a cell derived from the animal, wherein an enhanced level of ATP syntrhesis compared to the level of ATP synthesis of a Cbl-deficient animal to which the compound has not been administered indicates that the compound enhances ATP synthesis.

In an alternative embodiment, the invention provides a method of identifying a compound that enhances ATP synthesis comprising: (a) administering a compound to a non-human animal expressing a functional Cbl protein and determining the amount of ATP synthesis in the animal or a cell derived from the animal; (b) determining the amount of ATP synthesis in a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal or a cell derived therefrom; and (c) comparing the amount of ATP synthesis in the animals or cells at (a) and (b) wherein a comparable amount of fatty ATP synthesis between (a) and (b) indicates that the compound enhances ATP synthesis.

In an alternative embodiment, the present invention provides a method of identifying a compound that enhances ATP synthesis comprising determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein reduced ubiquitin ligase activity of the Cbl protein in the presence of the compound indicates that the compound enhances ATP synthesis .

In an alternative embodiment, the present invention provides a method for identifying a compound that enhances ATP synthesis comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein reduced phosphorylation of tyrosine residues in the Cbl protein in the presence of the compound indicates that the compound enhances ATP synthesis.

A further aspect of the present invention provides a method of identifying a compound that enhances glucose uptake such as, for example, in the treatment of hypolipidemia (eg. as observed in subjects suffering from abetalipoproteinemia, malnutrition or hematologic malignancies, such as acute myelocytic leukemia or chronic myelocytic leukemia), said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (b) determining the glucose uptake into liver, fat or muscle cells of the animal, wherein enhanced uptake compared to the glucose uptake into liver, fat or muscle cells of a Cbl-deficient animal to which the compound has not been administered indicates that the compound enhances glucose uptake.

In an alternative embodiment, the present invention provides a method of identifying a compound that enhances glucose uptake comprising determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein reduced ubiquitin ligase activity of the Cbl protein in the presence of the compound indicates that the compound reduces fat deposition or enhances glucose uptake.

In an alternative embodiment, the present invention provides a method for identifying a compound that enhances glucose uptake comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein reduced phosphorylation of tyrosine residues in the Cbl protein in the presence of the compound indicates that the compound enhances glucose uptake.

In an alternative embodiment, the invention provides a method of identifying a compound that reduces glucose uptake into liver, fat or muscle cells such as, for example, in the treatment of obesity, said method comprising: (a) administering a compound that enhances glucose uptake to a genetically modified non-human animal

comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal and determining the glucose uptake into liver, fat or muscle cells; (b) administering a compound to the animal and determining the glucose uptake into liver, fat or muscle cells of the animal, wherein a similar or reduced uptake at (b) compared to (a) indicates that the compound reduces glucose uptake into liver, fat or muscle cells.

In an alternative embodiment, the invention provides a method of identifying a compound that reduces glucose uptake into liver, fat or muscle cells comprising: (a) administering a compound to a non-human animal expressing a functional Cbl protein and determining the glucose uptake into liver, fat or muscle cells of the animal; (b) determining the glucose uptake into liver, fat or muscle cells of a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (c) comparing the glucose uptake into liver, fat or muscle cells of the animals at (a) and (b) wherein a comparable uptake between (a) and (b) indicates that the compound reduces glucose uptake into liver, fat or muscle cells.

In an alternative embodiment, the present invention provides a method of identifying a compound that reduces glucose uptake comprising determining the ubiquitin ligase activity of a Cbl protein in the presence and absence of the compound wherein enhanced ubiquitin ligase activity of the Cbl protein in the presence of the compound indicates that the compound reduces glucose uptake.

In an alternative embodiment, the present invention provides a method for identifying a compound that reduces glucose uptake comprising determining the level of tyrosine phosphorylation of a Cbl protein in the presence and absence of the compound wherein enhanced phosphorylation of tyrosine residues in the Cbl protein in the presence of the compound indicates that the compound reduces glucose uptake.

A further aspect of the present invention provides methods for determining a modulator of the activity, formation or stability of a protein complex selected from the group consisting of: (i) a Cbl-APS complex; (ii) a Cbl-CAP complex; (iii) a Cbl-CAP-

flotillin complex; (iv) a Cbl- C3G complex; (v) a Cbl-CrkII complex; and (vi) a Cbl-C3G-CrkII complex.

In a further aspect, the invention provides methods relating to the treatment of animal or human subjects wherein a compound that modulates (i.e. enhances or reduces or prevents) Cbl expression or activity is administered to the animal. Such methods apply *mutatis mutandis* to the treatment of a wide range of conditions associated with Cbl function, such as, for example, hyperglycemia, hyperinsulinemia, obesity, adult-onset obesity, aberrant fatty acid synthesis and/or fatty acid oxidation, non-insulin-dependent diabetes mellitus, type II diabetes, glucose intolerance, hypertrophy or hyperplasia of the islets of Langerhans, or for cosmetic purposes, such as, for example, bodybuilding or weight management (i.e. weight loss or weight gain). It is to be understood that such applications of the invention also relate to the productivity of stock and farm animals (e.g. dairy and beef cattle, pigs, horses, sheep, etc) such as, for example, for modulating the amount of fat they deposit or their ratio of fat to muscle mass. In accordance with this aspect of the invention, an amount of a Cbl agonist or antagonist is administered to the animal or human subject effective to modulate the expression or activity of Cbl in the subject.

In another embodiment, the invention also provides a method of treating a feeding disorder characterized by reduced dietary intake or suppressed appetite in a subject said method comprising administering to the subject an amount of a Cbl antagonist effective to enhance the appetite or dietary intake of the subject. The method of the invention is particularly suited to the treatment of anorexia or bulimia.

In a related embodiment, the invention also provides a method of treating a feeding disorder characterized by reduced dietary intake or suppressed appetite in a subject said method comprising administering to the subject an amount of a compound that reduces expression of functional Cbl effective to enhance the appetite or dietary intake of the subject.

A still further aspect of the present invention provides a method of modulating the amount of phosphorylated ACC and/or ACC activity in a cell or subject comprising modulating the activity or amount of a Cbl protein in the cell. In accordance with this

embodiment, modulation of the amount of phosphorylated ACC and/or ACC activity in a cell or subject may also cause modulation of fatty acid synthesis and/or fatty acid oxidation and/or ATP synthesis in said cell or subject.

Brief description of the drawings

Figure 1 is a graphical representation showing the body weight in grams (ordinate) of male (circles) and female (triangles) wild type mice (open symbols), and Cbl-deficient male (circles) and female (triangles) mice that are homozygous for a mutation in both alleles of the Cbl locus (filled symbols). Body weights of mice were determined every week from weaning to 16 weeks of age (x-axis). Data indicate that Cbl-deficient male mice have significantly higher body weight than their wild-type counterparts, throughout the time period tested. The weights of Cbl-deficient female mice are comparable to the weights of wild-type females.

Figure 2 is a graphical representation showing the dietary food intake in grams per day (ordinate) of male (circles) and female (triangles) wild type mice (open symbols), and Cbl-deficient male (circles) and female (triangles) mice that are homozygous for a mutation in both alleles of the Cbl locus (filled symbols). Food intake was determined every week from 5 weeks of age until 16 weeks of age (x-axis). Data indicate that Cbl-deficient male and female mice have significantly enhanced dietary intake (i.e. enhanced appetite) than their wild-type counterparts, throughout the time period tested. The dietary intake of Cbl-deficient female mice is also significantly higher than the dietary intake of wild-type males.

Figure 3 is a graphical representation showing the dietary food intake relative to body weight (ordinate) of male (circles) and female (triangles) wild type mice (open symbols), and Cbl-deficient male (circles) and female (triangles) mice that are homozygous for a mutation in both alleles of the Cbl locus (filled symbols). Food intake relative to body weight was determined every week from 5 weeks of age until 16 weeks of age (x-axis). Data indicate that Cbl-deficient male and female mice have significantly enhanced specific dietary intake (i.e. enhanced appetite as determined by food intake relative to body weight) than their wild-type counterparts, throughout the time period tested.

Figure 4 is a tabular representation showing the tissue weights (average +/- SEM) of wild type mice and Cbl-deficient mice that are homozygous for a mutation in both alleles of the Cbl locus (filled symbols). WAT, white adipose tissue; BAT, brown adipose tissue; QUAD, quadriceps muscle. Data indicate reduced adipose tissue weight for Cbl-deificient animals relative to wild type animals.

Figure 5 is a photographic representation showing adipocyte size in wild type mice (left) and Cbl-deficient mice that are homozygous for a mutation in both alleles of the Cbl locus (right).

Figure 6 is a tabular representation showing adipocyte diameter (μm) adipocyte volume (pl/cell) and lipid content (ng/cell) of male (Top panel) and female (Lower panel) wild type mice (WT), compared to male (Top panel) and female (Lower panel) Cbl-deficient mice that are homozygous for a mutation in both alleles of the Cbl locus (KO). Data indicate the mean values +/- SEM. Data indicate that for both males and females, there is a significant reduction in adipocyte size and volume, and reduced lipid content of adipocytes in Cbl-deficient mice relative to wild-type mice.

Figure 7A is a graphical representation showing glucose transport in soleus muscles of c-CBL$^{-/-}$ mice. Soleus muscles were removed from c-CBL-/- mice and wild-type c-CBL+/+ mice and incubated in the presence of labeled 2-deoxyglucose and no insulin (basal), 300μU/ml insulin (submax) and 1000μU/ml insulin (supramax). The muscle was then solubilized and the amount of labeled 2-deoxyglucose taken up by the muscle determined using a liquid scintillation counter. This amount +/- SEM was then graphically represented. *, $p<0.05$; *, $p<0.01$.

Figure 7B is a graphical representation showing glucose transport in extensor digitorum longus muscles of c-CBL$^{-/-}$ mice. Extensor digitorum longus muscles were removed from c-CBL-/- mice and wild-type c-CBL+/+ mice and incubated in the presence of labeled 2-deoxyglucose and no insulin (basal), 300μU/ml insulin (submax) and 1000μU/ml insulin (supramax). The muscle was then solubilized and the amount of labeled 2-deoxyglucose taken up by the muscle determined using a liquid scintillation counter. This amount +/- SEM was then graphically represented. *, $p<0.05$.

Figure 8 is a graphical representation showing glucose transport in fat explants taken from c-CBL$^{-/-}$ mice. Epididymal fat pads removed from c-CBL-/- mice and wild-type c-CBL+/+ mice and minced. Samples were then incubated in the presence of labeled 2-deoxyglucose and no insulin (0), 0.05nM insulin (0.05) and 1nM insulin (1). The amount of labeled 2-deoxyglucose taken up by the fat explant was determined using a liquid scintillation counter. This amount +/- SEM was then graphically represented.

Figure 9A is a graphical representation showing the amount of phosphorylated ACC in Cbl-/- mice. Protein derived from quadriceps of Cbl-/- (KO) and wild-type (WT) mice was resolved using SDS-PAGE and transferred to a membrane. The amount of phosphorylated ACC was then determined using an anti-phosphorylated ACC antibody. Results are presented as percentage of phosphorylated ACC detected in wild-type mice. *, $p<0.05$.

Figure 9B is a graphical representation showing the amount of AMP activated protein kinase (AMPK) in Cbl-/- mice. Protein derived from quadriceps of Cbl-/- (KO) and wild-type (WT) mice was resolved using SDS-PAGE and transferred to a membrane. The amount of AMPK was then determined using an anti-AMPK antibody. Results are presented as percentage of AMPK detected in wild-type mice.

Figure 9C is a graphical representation showing the amount of cytochrome-C (CytC) in Cbl-/- mice. Protein derived from quadriceps of Cbl-/- (KO) and wild-type (WT) mice was resolved using SDS-PAGE and transferred to a membrane. The amount of CytC was then determined using an anti-CytC antibody. Results are presented as percentage of CytC detected in wild-type mice. *, $p<0.05$.

Figure 9D is a graphical representation showing the amount of uncoupling protein 3 (UCP3) in Cbl-/- mice. Protein derived from quadriceps of Cbl-/- (KO) and wild-type (WT) mice was resolved using SDS-PAGE and transferred to a membrane. The amount of UCP3 was then determined using an anti-UCP3 antibody. Results are presented as percentage of UCP3 detected in wild-type mice. *, $p<0.05$.

Figure 9E is a graphical representation showing the amount of insulin receptor (IR) in Cbl-/- mice. Protein derived from quadriceps of Cbl-/- (KO) and wild-type (WT) mice was resolved using SDS-PAGE and transferred to a membrane. The amount of IR was then determined using an anti-IR antibody. Results are presented as percentage of IR detected in wild-type mice. *, $p<0.05$.

Figure 9F is a graphical representation showing the amount of glucose transporter 4 (GLUT4) in Cbl-/- mice. Protein derived from quadriceps of Cbl-/- (KO) and wild-type (WT) mice was resolved using SDS-PAGE and transferred to a membrane. The amount of GLUT4 was then determined using an anti-GLUT4 antibody. Results are presented as percentage of GLUT4 detected in wild-type mice. *, $p<0.05$.

Detailed description of the Preferred embodiments

One aspect of the present invention provides a method of identifying a compound that suppresses or reduces feeding behaviour, such as, for example, in the treatment of obesity, said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (b) determining the feeding behaviour of the animal, wherein reduced appetite or dietary intake of the animal compared to the appetite or dietary intake of a Cbl-deficient animal to which the compound has not been administered indicates that the compound suppresses or reduces feeding behaviour.

As used herein, the term "Cbl" shall be taken to mean any peptide, polypeptide, or protein having at least about 80% amino acid sequence identity to the amino acid sequence of a human or mouse c-Cbl polypeptide set forth in SEQ ID NO: 2 or 3. The term "Cbl" shall also be taken to include a peptide, polypeptide or protein having the known biological activity of Cbl, or the known binding specificity of Cbl including c-Cbl. For the purposes of nomenclature, the amino acid sequences of the murine and human Cbl polypeptides are exemplified herein, as SEQ ID Nos: 2 and 3, respectively. Preferably, the percentage identity to SEQ ID NO: 2 or 3 is at least about 85%, more preferably at least about 90%, even more preferably at least about 95% and still more preferably at least about 99%.

A "Cbl" protein generally comprises about 906 amino acid residues in length. The full-length protein generally comprises a tyrosine kinase binding domain that binds the protein to phosphotyrosine residues, thereby coupling Cbl to growth factor receptor signalling. For example, the c-Cbl is phosphorylated on 3-4 tyrosine residues in response to growth factors, the phosphorylated residues representing binding domains for SH2 domain containing proteins such as PI 3' kinase and Crk II. The tyrosine kinase binding domain generally will comprise a four helix bundle, an EF hand domain and an SH2 domain. Downstream of the tyrosine kinase binding domain is generally a C3HC4 RING finger domain having high sequence similarity to that found in ubiquitin ligase proteins. At the C terminal portion of the protein there is generally a proline rich region (PRR) which can bind SH3 domain-containing proteins (e.g. CAP). At the extreme C terminus of Cbl there is generally located a leucine zipper domain (b-Zip) and a ubiquitin association domain that regulates homodimerization of Cbl.

The various embodiments of the present invention directed to the identification of compounds that modulate feeding behaviour (e.g. in the treatment of obesity, anorexia or bulimia), fat deposition, metabolic rate, the ratio of lean muscle mass to body fat, or glucose uptake (e.g. in the treatment of obesity or type II diabetes), can also be carried out by determining a physical property of the Cbl protein, or alternatively, a catayltic activity of the Cbl protein, that is modified during glucose uptake into fat cells or muscle cells.

The ability of a compound to enhance feeding behaviour, reduce fat deposition, enhance metabolic rate, enhance the ratio of lean muscle mass to body fat, enhance ACC phosphorylation and/or reduce ACC activity, reduce fatty acid synthesis, enhance fatty acid oxidation or enhance ATP synthesis can thus be readily assayed by determining whether or not the compound reduces a Cbl activity that is disrupted in the Cbl-deficient mouse model. For example, reduced ubiquitin ligase activity of Cbl protein, or reduced phosphorylation of tyrosine residues in the Cbl protein (e.g. linked to growth receptor-mediated signalling events such as by measuring cAMP-mediated phosphorylation), or reduced binding of Cbl to an SH3-containing protein, would indicate the ability of a compound to produce these effects. Standard assays for determining tyrosine phosphorylation of Cbl, receptor-mediated signalling of tyrosine

phosphorylation in Cbl protein, ubiqitin ligase activity of Cbl or binding of Cbl to SH3 domains, are known to the skilled artisan.

Similarly, the ability of a compound to reduce feeding behaviour, enhance fat deposition, reduce metabolic rate, enhance the ratio of body fat to lean muscle mass, reduce ACC phosphorylation and/or enhance ACC activity, enhance fatty acid synthesis, reduce fatty acid oxidation, or reduce ATP synthesis can be assayed by determining whether or not the compound enhances ubiquitin ligase activity of Cbl protein, or enhances phosphorylation of tyrosine residues in the Cbl protein (e.g. linked to growth receptor-mediated signalling events such as by measuring cAMP-mediated phosphorylation), or enhances binding of Cbl to an SH3-containing protein.

By "Cbl-deficient" is meant that insufficient functional Cbl protein is produced to facilitate the level of glucose uptake or glycogen synthesis or lipogenesis detected in a wild type animal that does not suffer from a disorder of glucose metabolism. Glucose uptake, glycogen synthesis, or lipogenesis can readily be determined using known methods, such as, for example, by determining 2-deoxyglucose uptake into isolated liver, fat or muscle cells and/or glycogen synthesis and/or lipogenesis in the presence and absence of insulin as described by Lazar et al., J. Biol. Chem. 270: 20801-20807, 1995.

Preferably, the Cbl deficiency causes reduced Cbl expression at least in liver, fat or muscle cells of the genetically modified animal, more preferably in a tissue selected from the group consisting of adipose, skeletal muscle and cardiac muscle. Effects on Cbl expression in other cells or tissues, such as, for example, immune cells or brain, are not to be excluded from the scope of the present invention.

In one embodiment, the genetic modification capable of producing the Cbl deficient phenotype is selected from the group consisting of a deletion, an insertion, a substitution and an inversion of nucleotides in an allele of the Cbl locus. In another embodiment, the genetic modification is a deletion of a nucleotide sequence within two alleles of the Cbl locus, wherein the deletion results in an absence of expression of a functional or full-length Cbl protein by the animal. In a particularly preferred embodiment, the genetic modification comprises the deletion of a nucleotide sequence

encoding a protein-encoding portion of the Cbl gene sufficient to prevent Cbl function, or alternatively, the introduction of an in-frame stop codon at a location in the protein-encoding portion of the Cbl gene sufficient to prevent expression of functional Cbl by one or both Cbl-encoding alleles. Even more preferably, the genetic modification comprises the targeted disruption of one or two alleles at the Cbl locus produces a truncated Cbl protein comprising the amino acid sequence set forth in SEQ ID NO: 1.

Preferably, the genetic modification is in both alleles of the Cbl locus (i.e. the animal is homozygous for the genetic modification).

The non-human animal is any mammal, such as, for example, a rodent, dog, pig, bovine, sheep, horse or goat. In one embodiment, the animal is a rodent selected from the group consisting of rabbit, rat, guinea pig and mouse. Conveniently, the animal is a mouse.

The Cbl-deficient "control" animal employed in this context can be any other animal that has reduced expression of Cbl expression and need not be isogenic to the animal on which the compound was tested (i.e. the "test" animal). Preferably, the control and test animals express similar levels of functional Cbl. More preferably, the control and test animals are isogenic. Preferably, the appetite or dietary intake of the animal is modified to a level that is comparable to the appetite or dietary intake of a wild-type animal and does not completely suppress appetite or dietary intake if the animal.

By "feeding behaviour" is meant appetite or the amount of food consumption of the animal in a particular time interval, or its dietary intake expressed in absolute terms or alternatively, as a proportion of total body mass. Dietary intake will also be generally determined relative to a particular time interval (e.g. per hour, per day, etc). In most non-human animals, dietary intake is the preferred measure.

The range of compounds contemplated herein for modulating feeding behaviour include peptides, including peptides derived from Cbl and capable of complementing the Cbl-deficiency; non-Cbl peptides, such as, for example Cbl peptidomimetics; small organic molecules, such as, for example derived from publicly available combinatorial

libraries; and nucleic acids, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide.

In one embodiment, the subject method further comprises formulating the identified compound for administration to a non-human animal or a human. The formulations can be suitable for administration by injection by a subcutaneous, intravenous, intranasal, or intraperitoneal route. Alternatively, they can be suitable for oral administration in the form of feed additives, tablets, troches, etc.

The compounds are conveniently formulated in a suitable excipient or diluent, such as, for example, an aqueous solvent, non-aqueous solvent, non-toxic excipient, such as a salt, preservative, buffer and the like. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous solvents include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the formulation suitable for administration to the animal are adjusted according to routine skills in the art. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as

sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

Optionally, the formulation will also include a carrier, such as, for example, bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), ovalbumin, mouse serum albumin, rabbit serum albumin and the like. Means for conjugating peptides to carrier proteins are also well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

In another embodiment, the subject method further comprises producing or synthesizing the compound that is tested on the genetically modified animal.

Peptidyl compounds are conveniently made by standard peptide synthesis, such as the Merrifield method of synthesis (Merrifield, *J Am Chem Soc, 85*,:2149-2154, 1963) and the myriad of available improvements on that technology (see e.g., Synthetic Peptides: A User's Guide, Grant, ed. (1992) W.H. Freeman & Co., New York, pp. 382; Jones (1994) The Chemical Synthesis of Peptides, Clarendon Press, Oxford, pp. 230.); Barany, G. and Merrifield, R.B. (1979) in *The Peptides* (Gross, E. and Meienhofer, J. eds.), vol. 2, pp. 1-284, Academic Press, New York; Wünsch, E., ed. (1974) *Synthese von Peptiden in Houben-Weyls Metoden der Organischen Chemie* (Müler, E., ed.), vol. 15, 4th edn., Parts 1 and 2, Thieme, Stuttgart; Bodanszky, M. (1984) *Principles of Peptide Synthesis*, Springer-Verlag, Heidelberg; Bodanszky, M. & Bodanszky, A. (1984) *The Practice of Peptide Synthesis, Springer-Verlag,* Heidelberg; Bodanszky, M. (1985) *Int. J. Peptide Protein Res.* 25, 449-474.

Preferably, the peptide is synthesized on a solid phase support, such as, for example, a polystyrene gel bead comprising polystyrene cross-linked with divinylbenzene, preferably 1% (w.w) divinylbenzene, which is further swollen using lipophilic solvent, such as, for example dichloromethane or dimethylformamide (DMF). The polystyrene can be functionalized by addition of chloromethane or amino methyl groups. Alternatively, cross-linked and functionalized polydimethyl-acrylamide gel can be used once swollen and solvated using DMF or dipolar aprotic solvent. Other solid phase supports known to those skilled in the art can also be used for peptide synthesis, such as, for example, polyethylene glycol-derived bead produced by grafting polyethylene glycol to the surface of inert polystyrene beads. Preferred commercially available solid phase supports include PAL-PEG-PS, PAC-PEG-PS, KA, KR, or TGR (Applied Biosystems, CA 94404, USA).

For solid phase peptide synthesis, blocking groups that are stable to the repeated treatments necessary for removal of the amino blocking group of the growing peptide chain and for repeated amino acid couplings, are used for protecting the amino acid side-chains during synthesis and for masking undesired reactivity of the α-amino, carboxyl or side chain functional groups. Blocking groups (also called protecting groups or masking groups) thus protect the amino group of the amino acid having an activated carboxyl group that is involved in the coupling reaction, or protect the carboxyl group of the amino acid having an acylated amino group that is involved in the coupling reaction.

During synthesis, coupling occurs following removal of a blocking group without the disruption of a peptide bond, or any protecting group attached to another part of the peptide. Additionally, the peptide-resin anchorage that protects the C-terminus of the peptide is protected throughout the synthetic process until cleavage from the resin is required. Accordingly, by the judicious selection of orthogonally protected α-amino acids, amino acids are added at desired locations to a growing peptide whilst it is still attached to the resin.

Preferred amino blocking groups are easily removable but sufficiently stable to survive conditions for the coupling reaction and other manipulations, such as, for example,

modifications to the side-chain groups. In one embodiment, amino blocking groups are selected from the group consisting of: (i) a benzyloxycarbonyl group (Z or carbocenzoxy) that is removed easily by catalytic hydrogenation at room temperature and ordinary pressure, or using sodium in liquid ammonia and hydrobromic acid in acetic acid; (ii) a urethane derivative; (iii) a t-Butoxycarbonyl group (Boc) that is introduced using t-butoxycarbonyl azide or di-tert-butyldicarbonate and removed using mild acid such as, for example, trifluoroacetic acid (50% TFA in dichloromethane), or HCl in acetic acid/dioxane/ethylacetate; (iv) a 9-fluorenylmethyloxycarbonyl group (Fmoc) that is cleaved under mildly basic, non-hydrolytic conditions, such as, for example, using a primary or secondary amine (eg. 20% piperidine in dimethyl formamide); (v) a 2-(4-biphenylyl) propyl(2)oxycarbonyl group (Bpoc); (vi) a 2-nitro-phenylsulfenyl group (Nps); and (vii) a dithia-succionyl group (Dts). Boc is widely used to protect the N-terminus in Fmoc chemistry, or Fmoc is widely used to protect the N-terminus in Boc chemistry.

Side chain-protecting groups will vary for the functional side chains of the amino acids forming the peptide being synthesized. Side-chain protecting groups are generally based on the Bzl group or the tBu group. Amino acids having alcohols or carboxylic acids in the side-chain are protected as Bzl ethers, Bzl esters, cHex esters, tBu ethers, or tBu esters. Side-chain protection of Fmoc amino acids requires blocking groups that are ideally base stable and weak acid (TFA) labile. Many different protecting groups for peptide synthesis have been described (*see* The Peptides, Gross *et al.* eds., Vol. 3, Academic Press, New York, 1981). For example, the 4-methoxy-2,3,6-trimethylphenylsulfonyl (Nd- Mtr) group is useful for Arginine side-chain protection, however deprotection of Arg(Mtr) requires prolonged TFA treatment. A number of soft acid (TFA, thalium (III) trifluoroacetate/TFA) labile groups, or TFA stable but thalium (III) trifluoroacetate/TFA labile groups, or soft acid stable groups are used to protect Cystine.

The two most widely used protection strategies are the Boc/Bzl- and the Fmoc/tBu-strategies. In Boc/Bzl, Boc is used for amino protection and the side-chains of the various amino acids are protected using Bzl- or cHex-based protecting groups. A Boc group is stable under catalytic hydrogenation conditions and is used orthogonally along with a Z group for protection of many side chain groups. In Fmoc/tBu, Fmoc is

used for amino protection and the side-chains are protected with tBu-based protecting groups.

Alternatively, the peptidyl compound is produced by the recombinant expression of nucleic acid encoding the amino acid sequence of said peptide. Random peptide-encoding libraries are particularly preferred for such purposes, because they provide a wide range of different compounds to test. Alternatively, naturally-occurring nucleic acids can be screened. According to this embodiment, nucleic acid encoding the peptidyl compound is produced by standard oligonucleotide synthesis or derived from a natural source and cloned into a suitable expression vector in operable connection with a promoter or other regulatory sequence capable of regulating expression in a cell-free system or cellular system..

Oligonucleotides are preferably synthesized with linker or adaptor sequences at the 5'- and 3'-ends to facilitate subsequent cloning into a suitable vector system using standard techniques.

Placing a nucleic acid molecule under the regulatory control of, i.e., "in operable connection with", a promoter sequence means positioning said molecule such that expression is controlled by the promoter sequence, generally by positioning the promoter 5' (upstream) of the peptide-encoding sequence.

The prerequisite for producing intact peptides in bacteria such as *E. coli* is the use of a strong promoter with an effective ribosome binding site. Typical promoters suitable for expression in bacterial cells such as *E. coli* include, but are not limited to, the *lacz* promoter, temperature-sensitive λ_L or λ_R promoters, T7 promoter or the IPTG-inducible *tac* promoter. A number of other vector systems for expressing the nucleic acid molecule of the invention in *E. coli* are well-known in the art and are described, for example, in Ausubel *et al* (*In:* Current Protocols in Molecular Biology. Wiley Interscience, ISBN 047150338, 1987) or Sambrook *et al* (*In:* Molecular cloning, A laboratory manual, second edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989). Numerous plasmids with suitable promoter sequences for expression in bacteria and efficient ribosome binding sites have been described, such as for example, pKC30 (λ_L: Shimatake and Rosenberg, *Nature 292*, 128, 1981);

pKK173-3 (*tac*: Amann and Brosius, *Gene 40,* 183, 1985), pET-3 (T7: Studier and Moffat, *J. Mol. Biol. 189,* 113, 1986); the pBAD/TOPO or pBAD/Thio-TOPO series of vectors containing an arabinose-inducible promoter (Invitrogen, Carlsbad, CA), the latter of which is designed to also produce fusion proteins with thioredoxin to enhance solubility of the expressed protein; the pFLEX series of expression vectors (Pfizer Inc., CT, USA); or the pQE series of expression vectors (Qiagen, CA), amongst others.

Typical promoters suitable for expression in viruses of eukaryotic cells and eukaryotic cells include the SV40 late promoter, SV40 early promoter and cytomegalovirus (CMV) promoter, CMV IE (cytomegalovirus immediate early) promoter amongst others. Preferred vectors for expression in mammalian cells (eg. 293, COS, CHO, 10T cells, 293T cells) include, but are not limited to, the pcDNA vector suite supplied by Invitrogen, in particular pcDNA 3.1 myc-His-tag comprising the CMV promoter and encoding a C-terminal 6xHis and MYC tag; and the retrovirus vector pSRαtkneo (Muller *et al., Mol. Cell. Biol., 11,* 1785, 1991). The vector pcDNA 3.1 myc-His (Invitrogen) is particularly preferred for expressing peptides in a secreted form in 293T cells, wherein the expressed peptide or protein can be purified free of conspecific proteins, using standard affinity techniques that employ a Nickel column to bind the protein via the His tag.

A wide range of additional host/vector systems suitable for expressing peptides are available publicly, and described, for example, in Sambrook *et al* (*In:* Molecular cloning, A laboratory manual, second edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989).

Means for introducing the nucleic acid or a gene construct comprising same into a cell for expression are well-known to those skilled in the art. The technique used for a given organism depends on the known successful techniques. Means for introducing recombinant DNA into animal cells include microinjection, transfection mediated by DEAE-dextran, transfection mediated by liposomes such as by using lipofectamine (Gibco, MD, USA) and/or cellfectin (Gibco, MD, USA), PEG-mediated DNA uptake, electroporation and microparticle bombardment such as by using DNA-coated tungsten or gold particles (Agracetus Inc., WI, USA) amongst others.

Techniques for synthesizing small organic compounds will vary considerably depending upon the compound, however such methods will be well known to those skilled in the art. In one embodiment, informatics is used to select suitable chemical building blocks from known compounds, for producing a combinatorial library. For example, QSAR(Quantitative Structure Activity Relationship) modelling approach uses linear regressions or regression trees of compound structures to determine suitability. The software of the Chemical Computing Group, Inc.(Montreal, Canada) uses high-throughput screening experimental data on active as well as inactive compounds, to create a probabilistic QSAR model, which is subsequently used to select lead compounds. The Binary QSAR method is based upon three characteristic properties of compounds that form a "descriptor" of the likelihood that a particular compound will or will not perform a required function: partial charge, molar refractivity (bonding interactions), and logP (lipophilicity of molecule). Each atom has a surface area in the molecule and it has these three properties associated with it. All atoms of a compound having a partial charge in a certain range are determined and the surface areas (Van der Walls Surface Area descriptor) are summed. The binary QSAR models are then used to make activity models or ADMET models, which are used to build a combinatorial library. Accordingly, information from known appetite suppressants and non-suppressants, including lead compounds identified in initial screens, can be used to expand the list of compounds being screened to thereby identify highly active compounds.

Another embodiment of the invention provides a method of identifying a compound that enhances feeding behaviour, such as, for example, in the treatment of anorexia or bulimia, said method comprising: (a) administering a compound that suppresses appetite or dietary intake to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal and determining the feeding behaviour of the animal; (b) administering a compound to the animal and determining the feeding behaviour of the animal, wherein enhanced appetite or dietary intake at (b) compared to (a) indicates that the compound enhances feeding behaviour.

Naturally, the compound administered at (b) will be different to the compound at (a).

Preferably, the compound that suppresses appetite or dietary intake acts via a Cbl-mediated mechanism, which can be verified using the genetically modified Cbl-deficient animal, such as by determining the ability of the compound to suppress appetite or dietary intake of Cbl-deficient mice according to a method described herein. In this case, step (a) *supra* of administering a compound that suppresses appetite or dietary intake to the genetically modified non-human animal will comprise: (i) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (ii) determining the feeding behaviour of the animal, wherein reduced appetite or dietary intake of the animal compared to a Cbl-deficient animal to which the compound has not been administered indicates that the compound suppresses appetite or dietary intake.

In an alternative embodiment, the invention provides a method of identifying a compound that modulates feeding behaviour, such as, for example, in the treatment of anorexia or bulimia, said method comprising: (a) administering a compound to a non-human animal expressing a functional Cbl protein and determining the feeding behaviour of the animal; (b) determining the feeding behaviour of a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (c) comparing the feeding behaviour of the animals at (a) and (b) wherein a comparable feeding behaviour between (a) and (b) indicates that the compound modulates feeding behaviour.

In one embodiment, the subject method further comprises formulating the identified compound for administration to a non-human animal or a human as described *supra*.

In another embodiment, the subject method further comprises producing or synthesizing the compound that is tested on the genetically modified animal.

The range of compounds contemplated herein include Cbl inhibitory compounds or antagonists of a biological function of Cbl. In one embodiment, the compound is selected from the group consisting of: a peptide, including a peptide derived from Cbl

and capable of inhibiting, reducing or repressing a Cbl function, including binding to a protein selected from the group consisting of CAP, CrkII and C3G; a Cbl dominant-negative mutant; a non-Cbl peptide inhibitors of Cbl; an antibody or antibody fragment which binds to Cbl and inhibits a Cbl function; a small organic molecule, and nucleic acid, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide inhibitor, an antisense nucleic acid directed against Cbl-encoding mRNA, or an anti-Cbl ribozyme, or a small interfering RNA (RNAi) that targets Cbl gene expression.

The term "antisense nucleic acid" shall be taken to mean DNA or RNA molecule that is complementary to at least a portion of a specific mRNA molecule (Weintraub, Scientific American 262:40, 1990) and capable of interfering with a post-transcriptional event such as mRNA translation. Antisense oligomers complementary to at least about 15 contiguous nucleotides of Cbl-encoding mRNA are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target Cbl-producing cell. The use of antisense methods is well known in the art (Marcus-Sakura, Anal. Biochem. 172: 289, 1988). Preferred antisense nucleic acid will comprise a nucleotide sequence that is complementary to at least 15 contiguous nucleotides of a sequence encoding the amino acid sequence set forth in SEQ ID NO: 2 or 3.

As used herein, a "ribozyme" is a nucleic acid molecule having nuclease activity for a specific nucleic acid sequence. A ribozyme specific for Cbl-encoding mRNA, for example, binds to and cleaves specific regions of the mRNA, thereby rendering it untranslatable. To achieve specificity, preferred ribozymes will comprise a nucleotide sequence that is complementary to at least about 12-15 contiguous nucleotides of a sequence encoding the amino acid sequence set forth in SEQ ID NO: 2 or 3.

As used herein, the terms "small interfering RNA", and "RNAi" refer to homologous double stranded RNA (dsRNA) that specifically targets a gene product, thereby resulting in a null or hypomorphic phenotype. Specifically, the dsRNA comprises two short nucleotide sequences derived from the target RNA encoding Cbl and having self-complementarity such that they can anneal, and interfere with expression of a target gene, presumably at the post-transcriptional level. RNAi molecules are described by

Fire et al., Nature 391, 806-811, 1998, and reviewed by Sharp, Genes & Development, 13, 139-141, 1999).

The term "dominant-negative mutant" refers to a Cbl polypeptide that has been mutated from its natural state and that interacts with a protein that Cbl normally interacts with thereby preventing endogenous native Cbl from forming the interaction. Preferred dominant negative mutants will lack that portion of Cbl that interacts with a protein selected from the group consisting of CAP, CrkII and C3G.

The "antibodies" contemplated herein are immunoreactive with Cbl polypeptides or functional fragments thereof. Antibodies which consist essentially of pooled monoclonal antibodies with different epitope specificities, as well as distinct monoclonal antibody preparations are contemplated. Monoclonal antibodies are produced from fragments of the Cbl protein that comprise one or more B cell epitopes by methods well known to those skilled in the art (Kohler et al, Nature 256:495, 1975). The term "antibody" as used herein includes intact molecules as well as fragments thereof, such as Fab and $F(ab')_2$, Fv and single chain antibody fragments capable of binding an epitopic determinant of Cbl.

An "Fab fragment" consists of a monovalent antigen-binding fragment of an antibody molecule, and can be produced by digestion of a whole antibody molecule with the enzyme papain, to yield a fragment consisting of an intact light chain and a portion of a heavy chain.

An "Fab' fragment" of an antibody molecule can be obtained by treating a whole antibody molecule with pepsin, followed by reduction, to yield a molecule consisting of an intact light chain and a portion of a heavy chain. Two Fab' fragments are obtained per antibody molecule treated in this manner.

An "$F(ab')_2$ fragment" of an antibody consists of a dimer of two Fab' fragments held together by two disulfide bonds, and is obtained by treating a whole antibody molecule with the enzyme pepsin, without subsequent reduction. A $(Fab')_2$ fragment.

An "Fv fragment" is a genetically engineered fragment containing the variable region of a light chain and the variable region of a heavy chain expressed as two chains.

A "single chain antibody" (SCA) is a genetically engineered single chain molecule containing the variable region of a light chain and the variable region of a heavy chain, linked by a suitable, flexible polypeptide linker.

In one embodiment, peptidyl Cbl inhibitors are chemically or recombinantly synthesized as oligopeptides (approximately 10-25 amino acids in length) spanning the Cbl sequence (SEQ ID NO: 2 or 3). Alternatively, Cbl fragments are produced by digestion of native or recombinantly produced Cbl by, for example, using a protease, e.g., trypsin, thermolysin, chymotrypsin, or pepsin. Computer analysis (using commercially available software, e.g. MacVector, Omega, PCGene, Molecular Simulation, Inc.) is used to identify proteolytic cleavage sites. The proteolytic or synthetic fragments can comprise as many amino acid residues as are necessary to partially or completely inhibit Cbl function. Preferred fragments will comprise at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more amino acids in length.

In one embodiment, peptides are selected which contain a sufficient number of B cell epitopes to elicit antibodies when administered to a mammal. Such peptides can be identified by immunizing a mammal with the peptide, either alone or combined with or linked to an adjuvant (e.g., a hapten), and testing sera from the immunized animal for anti-Cbl antibodies. Preferred peptides generate anti-Cbl antibodies which inhibit a Cbl function.

Preferred peptidyl Cbl inhibitors will also not comprise a sufficient number of T cell epitopes to induce T-cell mediated (e.g., cytokine) responses when determined using any of a number of well known techniques, such as epitope prediction using algorithms (see e.g., Rothbard and Taylor EMBO J. 7: 93-100, 1988; Berzofsky, Philos Trans R. Soc. Lond. 323: 535-544, 1989; Rothbard, 1st Forum in Virology, Annals of the Pasteur Institute, pp 518-526, Dec. 1986; Rothbard and Taylor, Embo, 7: 93-100, 1988; EP 0 304 279; and Margalit et al., J. Immunol., 138: 2213-2229, 1987); or

screening of peptide inhibitors for human T cell stimulating activity or T cell proliferation assays (e.g. Proc. Natl. Acad. Sci USA, 86:1333, 1989).

Other preferred peptide inhibitors of Cbl are located on the surface of the Cbl proteins, e.g., hydrophilic regions, as well as regions with high antigenicity or fragments with high surface probability scores can be identified using computer analysis programs well known to those of skill in the art (Hopp and Wood, (1983), Mol.Immunol., 20, 483-9, Kyte and Doolittle, (1982), J. Mol. Biol., 157, 105-32, Corrigan and Huang, (1982), Comput. Programs Biomed, 3, 163-8).

Anti-Cbl antibodies or antibody fragments are generated using the entire Cbl polypeptide or an immunogenic fragment thereof (alone or linked to a suitable carrier or hapten) to immunize a subject (e.g., a mammal including, but not limited to a rabbit, goat, mouse or other mammal). For example, the methods described in U.S. Pat. Nos. 5,422,110; 5,837,268; 5,708,155; 5,723,129; and 5,849,531, can be used and are incorporated herein by reference. In a preferred embodiment, the mammal being immunized does not contain endogenous Cbl (e.g., a Cbl-deficient genetically modified animal). The immunogenic preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic proteolytic or synthetic Cbl peptide preparation induces a polyclonal anti-Cbl antibody response. The anti-Cbl antibody titer in the immunized subject is generally monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized Cbl. Subsequently, the sera from the immunized subjects can be tested for Cbl inhibitory activity.

Alternatively, is also possible to immunize the subject with nucleic acid expressing Cbl using DNA immunization technology, such as that disclosed in U.S. Pat. No. 5,795,872 to Ricigliano et al., or in U.S. Pat. No. 5,643,578 to Robinson et al.

The antibody molecules directed against Cbl can be isolated from the mammal (e.g., from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the anti-Cbl antibody titers are highest, antibody-producing cells can be

obtained from the subject and used to prepare e.g., monoclonal antibodies by standard techniques, such as the hybridoma technique originally described in the following disclosures: Kohler and Milstein Nature 256:495-497, 1975; Brown et al. J. Immunol. 127:53946, 1981; Brown et al. J. Biol Chem.255: 4980-4983, 1980; Yeh et al. Proc. Natl. Acad. Sci. USA 76:2927-2931, 1976; Yeh et al. Int. J. Cancer 29: 269-275, 1982; Kozbor et al. Immunol Today 4:72, 1983; Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1985. The technology for producing monoclonal antibody hybridomas is well known in the art. Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a Cbl peptide immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds Cbl.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-Cbl monoclonal antibody (see, e.g., G. Galfre et al., Nature 266: 550-552, 1970). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, e.g., the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind Cbl, e.g., using a standard ELISA assay. The antibodies can then be tested for Cbl inhibitory activity.

A further aspect of the present invention provides a method of identifying a compound that enhances fat deposition or reduces lean muscle mass or enhances the ratio of body fat to muscle or reduces metabolic rate, such as, for example, in the treatment of hypolipidemia (eg. as observed in subjects suffering from abetalipoproteinemia, malnutrition or hematologic malignancies, such as acute myelocytic leukemia or chronic myelocytic leukemia), said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (b) determining the fat content of the animal, wherein enhanced fat content of the animal compared to the fat content of a Cbl-deficient animal to which the compound has not been administered indicates that the compound enhances fat deposition or reduces lean muscle mass or enhances the ratio of body fat to muscle or reduces metabolic rate.

By "fat deposition" is meant the amount of fat deposited or the rate at which fat is deposited in liver, fat or muscle cells. Standard means known to the skilled artisan are used to determine fat deposition. "Lean muscle mass" means muscle tissue that is substantially fat-free. The "ratio of body fat to muscle mass" means the relative amount of total body fat to total non-fat tissue. "Metabolic rate" means the ability of a subject to utilize dietary intake for immediate energy needs, rather than store such dietary intake as body fat.

The genetically modified animal and the Cbl-deficient animal are described *supra*.

The range of compounds contemplated herein for enhancing fat deposition or reducing lean muscle mass or enhancing the ratio of body fat to muscle or reducing metabolic rate include peptides, including peptides derived from Cbl and capable of complementing the Cbl-deficiency; non-Cbl peptides, such as, for example Cbl peptidomimetics; small organic molecules, such as, for example derived from publicly available combinatorial libraries; and nucleic acids, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide. Such compounds are described *supra*.

In one embodiment, the subject method further comprises formulating the identified compound for administration to a non-human animal or a human. Such formulations are described *supra*.

In another embodiment, the subject method further comprises producing or synthesizing the compound that is tested on the genetically modified animal. Such methods are described *supra*.

In an alternative embodiment, the invention provides a method of identifying a compound that reduces fat deposition or enhances lean muscle mass or reduces the ratio of body fat to muscle or enhances metabolic rate, such as, for example, in the treatment of obesity, said method comprising: (a) administering a compound that enhances fat deposition or glucose uptake to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal and determining the fat content of the animal; (b) administering a compound to the animal and determining the fat content of the animal, wherein a similar or reduced fat content at (b) compared to (a) indicates that the compound reduces fat deposition or enhances lean muscle mass or reduces the ratio of body fat to muscle or enhances metabolic rate.

Preferably, the animal is maintained on a diet comprising high glycemic index food, such as, for example, carrots or food supplemented with sucrose.

Preferably, the compound that enhances fat deposition, or reduces lean muscle mass or enhances the ratio of body fat to muscle or reduces metabolic rate acts via a Cbl-mediated mechanism, which can be verified using the genetically modified Cbl-deficient animal, such as by determining the ability of the compound to enhance fat content of Cbl-deficient mice according to a method described herein. In this case, step (a) *supra* of administering a compound that enhances fat deposition or glucose uptake comprises (i) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (ii) determining the fat content of the animal, wherein enhanced fat content of the

animal compared to a Cbl-deficient animal to which the compound has not been administered indicates that the compound enhances fat deposition, or reduces lean muscle mass or enhances the ratio of body fat to muscle or reduces metabolic rate.

In an alternative embodiment, the invention provides a method of identifying a compound that reduces fat deposition or enhances lean muscle mass or reduces the ratio of body fat to muscle or enhances metabolic rate comprising: (a) administering a compound to a non-human animal expressing a functional Cbl protein and determining the fat content of the animal; (b) determining the fat content of a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (c) comparing the fat contents of the animals at (a) and (b) wherein a comparable fat content between (a) and (b) indicates that the compound reduces fat deposition or enhances lean muscle mass or reduces the ratio of body fat to muscle or enhances metabolic rate.

Preferably, the animals are maintained on similar or identical diets, more preferably, on diets comprising high glycemic index food.

The range of compounds contemplated herein include Cbl inhibitory compounds or antagonists of a biological function of Cbl. In one embodiment, the compound is selected from the group consisting of: a peptide, including a peptide derived from Cbl and capable of inhibiting, reducing or repressing a Cbl function, including binding to a protein selected from the group consisting of CAP, CrkII and C3G; a Cbl dominant-negative mutant; a non-Cbl peptide inhibitors of Cbl; an antibody or antibody fragment which binds to Cbl and inhibits a Cbl function; a small organic molecule, and nucleic acid, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide inhibitor, an antisense nucleic acid directed against Cbl-encoding mRNA, an anti-Cbl ribozyme, RNAi or siRNA.

In one embodiment, the subject method further comprises formulating the identified compound for administration to a non-human animal or a human. Such formulations are described *supra*.

In another embodiment, the subject method further comprises producing or synthesizing the compound that is tested on the genetically modified animal. Such methods are described *supra*.

A further aspect of the present invention provides a method of identifying a compound that reduces the level of phosphorylated ACC and/or enhances ACC activity in a cell or subject, such as, for example, in the treatment of hypolipidemia (eg. as observed in subjects suffering from abetalipoproteinemia, malnutrition or hematologic malignancies, such as acute myelocytic leukemia or chronic myelocytic leukemia), said method comprising: (a) administering a compound to a genetically modified non-human animal or cell derived therefrom comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (b) determining the level of ACC phosphorylation and/or ACC activity of the animal, wherein enhanced ACC activity and/or reduced levels of phosphorylated ACC in the animal or cell compared to the level of phosphorylated ACC and/or ACC activity in a Cbl-deficient animal or cell derived therefrom to which the compound has not been administered indicates that the compound the level of phosphorylated ACC and/or enhances ACC activity.

As used herein the term "ACC" or "acetyl CoA carboxylase" shall be taken to include all forms of acetyl CoA carboxylase, for example, an ACC1 or an ACC2 polypeptide. Preferably, an ACC is an ACC2 protein. Even more preferably, the term "ACC" shall be taken to mean any peptide, polypeptide or protein having at least about 80% amino acid sequence identity to the amino acid sequence of human ACC2 set forth in SEQ ID NO: 4 or a fragment thereof. The term "ACC" shall also be taken to include a peptide, polypeptide or protein having a known biological activity of an ACC.

An ACC protein, and, in particular, an ACC2 protein comprises a signal peptide targeting the ACC protein to a mitochondrial membrane. Without been bound by theory it is thought that ACC2 produces malonyl-CoA that is a regulator of the carnitine/palmitoyl-CoA shuttle system associated with the mitochondrial membrane.

By "phosphorylated ACC" is meant that an ACC polypeptide as described herein has had a phosphate group covalently attached to one or more amino acids within the

polypeptide. Preferably, the phosphate group is attached to one or more serine residues within the ACC polypeptide.

Methods of detecting a phosphorylated protein or determining the level of a phosphorylated protein are known in the art. As exemplified herein, a phosphorylated ACC specific antibody is used to detect the amount of phosphorylated ACC in a sample. A polyclonal anti-phosphorylated ACC antibody is available from Cell Signalling Technologies Inc. (Beverly, MA, USA) or from Upstate Biochemicals (Lake Placid, NY, USA). Preferably, the amount of phosphorylated ACC is determined using an ELISA assay as described herein, however, the amount of phosphorylated ACC may be determined using any method known in the art, and/or described, for example, in Scopes (In: Protein Purification: Principles and Practice, Third Edition, Springer Verlag, 1994).

As will be apparent to the skilled artisan any method that detects the amount of a protein in a sample using an antibody or ligand may be adapted to detect the amount of a phosphorylated protein in a sample, eg. Western blotting, immunohistochemstry, immunofluorescence, RIA, EIA, a method utilizing a biosensor or a method using a biochip. Clearly such methods are encompassed by the instant invention

As phosphorylation of ACC regulates the activity of ACC, the amount of phosphorylation may be determined by detecting ACC activity in a sample. Methods of detecting ACC activity are known in the art. Such methods usually require the measurement of a protein or biochemical process that is regulated by ACC. For example, ACC2 produces malonyl-CoA that inhibits carnitine palmitoyl-CoA transferase 1 (CPT1) activity. Cakes *et al*, *J. Exp. Zool. 281*:6-11 describe an assay for the measurement of CPT1 activity that may be used to determine ACC activity.

Alternatively, ACC activity is known to regulate the expression of several proteins invoved in glucose metabolism. Accordingly, an assay that determines the expression of such a protein may be used to determine ACC activity. As exemplified herein, increased phosphorylation of ACC is associated with increased expression of UCP3, cytochrome C, insulin receptor and GLUT4. Accordingly, an assay that determines the

level of expression of a gene selected from the group consisiting of UCP3, cytochrome C, insulin receptor and GLUT4 is used to determine ACC activity.

Methods of determining the expression of a gene product (ie. mRNA or protein) are known in the art, such as, for example, *in situ* hybridization, quantitative PCR and microarray technology. All such assay formats are encompassed by the present invention.

In a preferred embodiment, the level of nucleic acid is determined by hybridizing a nucleic acid probe to a nucleic acid in a sample under at least low stringency hybridization conditions and detecting the hybridization using a detection means.

For nucleic acid hybridization-based approaches, shorter probes are hybridized at lower stringency hybridization (ie. reduced temperature and/or higher salt concentration and/or higher detergent concentration) than longer nucleic acid probes. Generally, hybridization is carried out well below the calculated melting temperature (Tm) of a DNA duplex comprising the probe. For example, the oligonucleotide probes exemplified herein have calculated Tm values in the range of about 55°C to about 60° C, suggesting that hybridization involving such probes should be carried out at a temperature in the range of ambient temperature to about 45°C, and more preferably between about 40°C to about 45°C (ie. low stringency to moderate stringency conditions). This contrasts with standard hybridization temperatures of about 65°C for nucleic acid probes of about 100 nucleotides or longer (ie. moderate to high stringency hybridization conditions).

For the purposes of defining the level of stringency to be used in these diagnostic assays, a low stringency is defined herein as being a hybridization and/or a wash carried out in 6xSSC buffer, 0.1% (w/v) SDS at 28°C, or equivalent conditions. A moderate stringency is defined herein as being a hybridization and/or washing carried out in 2xSSC buffer, 0.1% (w/v) SDS at a temperature in the range 45°C to 65°C, or equivalent conditions. A high stringency is defined herein as being a hybridization and/or wash carried out in 0.1xSSC buffer, 0.1% (w/v) SDS, or lower salt concentration, and at a temperature of at least 65°C, or equivalent conditions.

Reference herein to a particular level of stringency encompasses equivalent conditions using wash/hybridization solutions other than SSC known to those skilled in the art.

Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS and/or increasing the temperature of the hybridization and/or wash. Those skilled in the art will be aware that the conditions for hybridization and/or wash may vary depending upon the nature of the hybridization matrix used to support the sample DNA, or the type of hybridization probe used.

In one embodiment, the sample or the probe is immobilized on a solid matrix or surface (e.g., nitrocellulose or a microtitre plate). For high throughput screening, the sample or probe will generally comprise an array of nucleic acids on glass or other solid matrix, such as, for example, as described in WO 96/17958. Techniques for producing high density arrays are described, for example, by Fodor *et al.*, Science 767-773, 1991, and in U.S. Pat. No. 5,143,854. Typical protocols for other assay formats can be found, for example in Current Protocols In Molecular Biology, Unit 2 (Northern Blotting), Unit 4 (Southern Blotting), and Unit 18 (PCR Analysis), Frederick M. Ausubul *et al.* (ed)., 1995.

The detection means according to this aspect of the invention may be any nucleic acid-based detection means such as, for example, nucleic acid hybridization or amplification reaction (eg. PCR), a nucleic acid sequence-based amplification (NASBA) system, inverse polymerase chain reaction (iPCR), or *in situ* polymerase chain reaction.

The probe can be labelled with a reporter molecule capable of producing an identifiable signal (e.g., a radioisotope such as ^{32}P or ^{35}S, or a fluorescent or biotinylated molecule, or a coloured dye e.g. TAMRA, FAM, ROC, etc). According to this embodiment, those skilled in the art will be aware that the detection of said reporter molecule provides for identification of the probe and that, following the hybridization reaction, the detection of the corresponding nucleotide sequences in the sample is facilitated. Additional probes can be used to confirm the assay results obtained using a single probe.

Wherein the detection means is an amplification reaction such as, for example, a polymerase chain reaction or a nucleic acid sequence-based amplification (NASBA) system or a variant thereof, one or more nucleic acid probes molecules of at least about 20 contiguous nucleotides in length is hybridized to genomic DNA and nucleic acid copies of the template are enzymically-amplified.

Those skilled in the art will be aware that there must be a sufficiently high percentage of nucleotide sequence identity between the probes and the nucleotide sequence of the sample template molecule for hybridization to occur. As stated previously, the stringency conditions can be selected to promote hybridization.

In one format, PCR provides for the hybridization of non-complementary probes to different strands of a double-stranded nucleic acid template molecule (ie. a DNA/DNA template), such that the hybridized probes are positioned to facilitate the 5'-to 3' synthesis of nucleic acid in the intervening region, under the control of a thermostable DNA polymerase enzyme. In accordance with this embodiment, one sense probe and one antisense probe as described herein is used.

Variations of the embodiments described herein are described in detail by McPherson *et al.*, PCR: A Practical Approach. (series eds, D. Rickwood and B.D. Hames), IRL Press Limited, Oxford. pp1-253, 1991.

Alternatively, the level of a protein encoded by a gene that is regulated by ACC activity in a sample is determined. Preferably an immunoassay is used to detect the amount of a protein in a sample, more preferably an ELISA assay as described herein. Antibodies particularly useful for detecting expression of ACC regulated genes are available commercially, for example, an anti-cytochrome C antibody is available from Research Diagnostics, Flanders, NJ, USA. An anti-UCP antibody is available from USBiologicals, Swampscott, USA. An anti-insulin receptor antibody is available from Chemicon, Temecula, CA, USA. An anti-GLUT4 antibody is available from Novus Biologicals, Littleton, CO, USA.

Alternative methods of detecting the amount of protein in a sample are known in the art and/or described herein.

The genetically modified animal and the Cbl-deficient animal are described *supra.*

The range of compounds contemplated herein for enhancing ACC phosphorylation or reducing ACC activity include peptides, including peptides derived from Cbl and capable of complementing the Cbl-deficiency; non-Cbl peptides, such as, for example Cbl peptidomimetics; small organic molecules, such as, for example derived from publicly available combinatorial libraries; and nucleic acids, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide. Such compounds are described *supra.*

In one embodiment, the subject method further comprises formulating the identified compound for administration to a non-human animal or a human. Such formulations are described *supra.*

In another embodiment, the subject method further comprises producing or synthesizing the compound that is tested on the genetically modified animal. Such methods are described *supra.*

In an alternative embodiment, the invention provides a method of identifying a compound that enhances the amount of a phosphorylated acetyl CoA carboxylase (ACC) enzyme and/or reduces the activity of an ACC enzyme in a cell , such as, for example, in the treatment of obesity or neurodegenerative disorders or for cosmetic purposes such as bodybuilding or weight loss, said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic mutation within an allele of its Cbl locus that reduces or prevents expression of functional Cbl in said animal; and (b) determining the activity and/or amount of phosphorylated ACC enzyme in the animal, wherein an enhanced level of phosphorylated enzyme and/or reduced activity of an ACC enzyme compared to the level of phosphorylated enzyme and/or activity of an ACC enzyme of a Cbl-deficient animal to which the compound has not been administered indicates that the compound enhances the amount of phosphorylated ACC enzyme and/or reduces the activity of an ACC enzyme.

Preferably, the compound that enhances ACC phosphorylation or reduces ACC activity acts via a Cbl-mediated mechanism, which can be verified using the genetically modified Cbl-deficient animal, such as by determining the ability of the compound to enhance ACC phosphorylation or reduces ACC activity of Cbl-deficient mice according to a method described herein. In this case, step (a) *supra* of administering a compound that enhances ACC phosphorylation or reduces ACC activity comprises (i) administering a compound to a genetically modified non-human animal or a cell derived therefrom comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (ii) determining the ACC phosphorylation or ACC activity in the animal or a cell derived therefrom, wherein enhanced ACC phosphorylation or reduced ACC activity of the animal compared to a Cbl-deficient animal to which the compound has not been administered indicates that the compound enhance ACC phosphorylation or reduces ACC activity .

In an alternative embodiment, the invention provides a method of identifying a compound that enhances the amount of a phosphorylated ACC enzyme and/or reduces the activity of an ACC enzyme comprising: (a) administering a compound to a non-human animal expressing a functional Cbl protein and determining the activity and/or amount of phosphorylated ACC enzyme; (b) determining the activity and/or amount of phosphorylated ACC enzyme in a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (c) comparing the activity and/or amount of phosphorylated ACC enzyme in the animals at (a) and (b) wherein a comparable activity and/or amount of phosphorylated ACC enzyme between (a) and (b) indicates that the compound enhances the amount of a phosphorylated ACC enzyme and/or reduces the activity of an ACC enzyme.

The range of compounds contemplated herein include Cbl inhibitory compounds or antagonists of a biological function of Cbl. In one embodiment, the compound is selected from the group consisting of: a peptide, including a peptide derived from Cbl and capable of inhibiting, reducing or repressing a Cbl function, including binding to a protein selected from the group consisting of CAP, CrkII and C3G; a Cbl dominant-negative mutant; a non-Cbl peptide inhibitors of Cbl; an antibody or antibody fragment

which binds to Cbl and inhibits a Cbl function; a small organic molecule, and nucleic acid, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide inhibitor, an antisense nucleic acid directed against Cbl-encoding mRNA, an anti-Cbl ribozyme, RNAi or siRNA.

In one embodiment; the subject method further comprises formulating the identified compound for administration to a non-human animal or a human. Such formulations are described *supra*.

In another embodiment, the subject method further comprises producing or synthesizing the compound that is tested on the genetically modified animal. Such methods are described *supra*.

A further aspect of the present invention provides a method of identifying a compound that reduces fatty acid oxidation and/or enhances fatty acid synthesis, such as, for example, in the treatment of hypolipidemia (eg. as observed in subjects suffering from abetalipoproteinemia, malnutrition or hematologic malignancies, such as acute myelocytic leukemia or chronic myelocytic leukemia), said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (b) determining the amount of fatty acid synthesis and/or fatty acid oxidation in the animal or a cell derived from the animal, wherein a reduced amount of fatty acid oxidation and/or a reduced amount of fatty acid synthesis compared to the amount of acid oxidation and/or amount of fatty acid synthesis of a Cbl-deficient animal to which the compound has not been administered indicates that the compound reduces fatty acid oxidation and/or enhances fatty acid synthesis.

Methods of determining fatty acid synthesis are known in the art and include, for example, incubating a cell lysate, eg. derived from a genetically modified organism, with carbon ([14C]malonyl-CoA) and either NADH or NAPDH, with or without putative modulatory compound. Incorporation of malonyl-CoA into fatty acids was measured by liquid scintillation counting

Methods of determining Fatty acid oxidation are known in the art and include, for example, measuring β-oxidation of fatty acids using [^{3}H]-palmitate as a substrate in a muscle or a hepatocyte isolated from a subject (eg. a genetically modified organism) essentially as described by Alam and Saggerson, *Biochem. J.*, *334*: 233-241, 1998 or Moon and Rhead, *J. Clin. Invest.*, *79*: 59-64.

The genetically modified animal and the Cbl-deficient animal are described *supra*.

The range of compounds contemplated herein for enhancing fatty acid synthesis and/or reducing fatty acid oxidation include peptides, including peptides derived from Cbl and capable of complementing the Cbl-deficiency; non-Cbl peptides, such as, for example Cbl peptidomimetics; small organic molecules, such as, for example derived from publicly available combinatorial libraries; and nucleic acids, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide. Such compounds are described *supra*.

In one embodiment, the subject method further comprises formulating the identified compound for administration to a non-human animal or a human. Such formulations are described *supra*.

In another embodiment, the subject method further comprises producing or synthesizing the compound that is tested on the genetically modified animal. Such methods are described *supra*.

In an alternative embodiment, the invention provides a method of identifying a compound that reduces fatty acid synthesis and/or enhances fatty acid oxidation, such as, for example, in the treatment of obesity, said method comprising: (a) administering a compound that enhances fatty acid oxidation and/or reduces fatty acid synthesis uptake to a genetically modified non-human animal or cell derived therefrom comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal and determining the amount of fatty acid synthesis and/or fatty acid oxidation; (b) administering a compound to the animal and determining the fatty acid synthesis and/or fatty acid oxidation of the animal or cell derived therefrom, wherein a similar or reduced level of fatty acid synthesis and/or fatty acid oxidation at (b) compared to (a)

indicates that the compound reduces fatty acid synthesis and/or enhances fatty acid oxidation.

Preferably, the compound that enhances fatty acid oxidation and/or reduces fatty acid synthesis acts via a Cbl-mediated mechanism, which can be verified using the genetically modified Cbl-deficient animal, such as by determining the ability of the compound to enhance fatty acid oxidation and/or reduce fatty acid syntheis of Cbl-deficient mice according to a method described herein. In this case, step (a) *supra* of administering a compound that enhances fatty acid oxidation and/or reduces fatty acid syntheis comprises (i) administering a compound to a genetically modified non-human animal or cell derived therefrom comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (ii) determining the amount of fatty acid oxidation and/or fatty acid synthesis, wherein enhanced fatty acid oxidation and/or reduced fatty acid synthesis of the animal or cell derived therefrom compared to a Cbl-deficient animal or cell derived therefrom to which the compound has not been administered indicates that the compound enhances fatty acid synthesis and/or reduces fatty acid synthesis.

In an alternative embodiment, the invention provides a method of identifying a compound that reduces fatty acid synthesis and/or enhances fatty acid oxidation: (a) administering a compound to a non-human animal or cell derived therefrom expressing a functional Cbl protein and determining the amount of fatty acid synthesis and/or fatty acid oxidation of the animal; (b) determining the fatty acid synthesis and/or fatty acid oxidation of a genetically modified non-human animal or cell derived therefrom comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (c) comparing the level of fatty acid synthesis and/or fatty acid oxidation of the animals at (a) and (b) wherein a comparable level of fatty acid synthesis and/or fatty acid oxidation between (a) and (b) indicates that the compound reduces fatty acid synthesis and/or enhances fatty acid oxidation.

The range of compounds contemplated herein include Cbl inhibitory compounds or antagonists of a biological function of Cbl. In one embodiment, the compound is

selected from the group consisting of: a peptide, including a peptide derived from Cbl and capable of inhibiting, reducing or repressing a Cbl function, including binding to a protein selected from the group consisting of CAP, CrkII and C3G; a Cbl dominant-negative mutant; a non-Cbl peptide inhibitors of Cbl; an antibody or antibody fragment which binds to Cbl and inhibits a Cbl function; a small organic molecule, and nucleic acid, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide inhibitor, an antisense nucleic acid directed against Cbl-encoding mRNA, an anti-Cbl ribozyme, RNAi or siRNA.

In one embodiment, the subject method further comprises formulating the identified compound for administration to a non-human animal or a human. Such formulations are described *supra*.

In another embodiment, the subject method further comprises producing or synthesizing the compound that is tested on the genetically modified animal. Such methods are described *supra*.

A further aspect of the present invention provides a method of identifying a compound that reduces ATP synthesis said method comprising: (a) administering a compound to a genetically modified non-human animal or cell derived therefrom comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (b) determining the amount of ATP synthesis in the animal or a cell derived from the animal, wherein a reduced amount of ATP synthesis compared to the amount of ATP synthesis of a Cbl-deficient animal to which the compound has not been administered indicates that the compound reduces ATP synthesis.

Methods of determining ATP synthesis are known in the art and are described, for example, in Manfredi *et al., Methods Cell Biol.*, 65: 133-145, 2001. Preferably, such an assay is performed using a cell or cell lysate derived from a subject, such as, for example a genetically modified animal subject.

The genetically modified animal and the Cbl-deficient animal are described *supra*.

The range of compounds contemplated herein for reducing ATP synthesis include peptides, including peptides derived from Cbl and capable of complementing the Cbl-deficiency; non-Cbl peptides, such as, for example Cbl peptidomimetics; small organic molecules, such as, for example derived from publicly available combinatorial libraries; and nucleic acids, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide. Such compounds are described *supra*.

In one embodiment, the subject method further comprises formulating the identified compound for administration to a non-human animal or a human. Such formulations are described *supra*.

In another embodiment, the subject method further comprises producing or synthesizing the compound that is tested on the genetically modified animal. Such methods are described *supra*.

In an alternative embodiment, the invention provides a method of identifying a compound that enhances ATP synthesis, such as, for example, in the treatment of obesity, said method comprising: (a) administering a compound that enhances ATP synthesis uptake to a genetically modified non-human animal or cell derived therefrom comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal and determining the amount of fatty acid synthesis and/or fatty acid oxidation; (b) administering a compound to the animal and determining the level of ATP synthesis in the animal or cell derived therefrom, wherein a similar or enhanced level of ATP synthesis level at (b) compared to (a) indicates that the compound enahnces ATP synthesis.

Preferably, the compound that enhances ATP synthesis acts via a Cbl-mediated mechanism, which can be verified using the genetically modified Cbl-deficient animal, such as by determining the ability of the compound to enhance ATP syntheis of Cbl-deficient mice or a cell derived therefrom according to a method described herein. In this case, step (a) *supra* of administering a compound that enhances ATP syntheis comprises (i) administering a compound to a genetically modified non-human animal or cell derived therefrom comprising a genetic modification within an allele of its Cbl

locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (ii) determining the amount of ATP synthesis, wherein enhanced ATP synthesis in the animal or cell derived therefrom compared to a Cbl-deficient animal or cell derived therefrom to which the compound has not been administered indicates that the compound enhances ATP synthesis.

In an alternative embodiment, the invention provides a method of identifying a compound that enhances ATP synthesis comprising: (a) administering a compound to a non-human animal or cell derived therefrom expressing a functional Cbl protein and determining the amount of ATP in the animal or cell; (b) determining the ATP synthesis of a genetically modified non-human animal or cell derived therefrom comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (c) comparing the level of ATP synthesis in the animals or cells at (a) and (b) wherein a comparable level of ATP synthesis between (a) and (b) indicates that the compound enhances ATP synthesis.

The range of compounds contemplated herein include Cbl inhibitory compounds or antagonists of a biological function of Cbl. In one embodiment, the compound is selected from the group consisting of: a peptide, including a peptide derived from Cbl and capable of inhibiting, reducing or repressing a Cbl function, including binding to a protein selected from the group consisting of CAP, CrkII and C3G; a Cbl dominant-negative mutant; a non-Cbl peptide inhibitors of Cbl; an antibody or antibody fragment which binds to Cbl and inhibits a Cbl function; a small organic molecule, and nucleic acid, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide inhibitor, an antisense nucleic acid directed against Cbl-encoding mRNA, an anti-Cbl ribozyme, RNAi or siRNA.

In one embodiment, the subject method further comprises formulating the identified compound for administration to a non-human animal or a human. Such formulations are described *supra*.

In another embodiment, the subject method further comprises producing or synthesizing the compound that is tested on the genetically modified animal. Such methods are described *supra*.

A further aspect of the present invention provides a method of identifying a compound that enhances glucose uptake such as, for example, in the treatment of hypolipidemia (eg. as observed in subjects suffering from abetalipoproteinemia, malnutrition or hematologic malignancies, such as acute myelocytic leukemia or chronic myelocytic leukemia), said method comprising: (a) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (b) determining the glucose uptake into liver, fat or muscle cells of the animal, wherein enhanced uptake compared to the glucose uptake into liver, fat or muscle cells of a Cbl-deficient animal to which the compound has not been administered indicates that the compound enhances glucose uptake.

Means for determining glucose uptake are well known in the art. Preferably, the process is performed *ex vivo* using liver, fat or muscle cells that have been previously isolated from the animal. In one embodiment, the glucose uptake is basal glucose uptake (i.e. glucose uptake measured in the absence of exogenously administered insulin). In another embodiment, the glucose uptake is insulin-mediated glucose uptake (i.e. glucose uptake measured following administration of insulin). All embodiments of hte invention described herein apply *mutatis mutandis* to both basal glucose uptake and insulin-mediated glucose uptake unless otherwise stated.

The genetically modified animal and the Cbl-deficient animal are described *supra*.

The range of compounds contemplated herein for enhancing glucose uptake include peptides, including peptides derived from Cbl and capable of complementing the Cbl-deficiency; non-Cbl peptides, such as, for example Cbl peptidomimetics; small organic molecules, such as, for example derived from publicly available combinatorial libraries; and nucleic acids, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide. Such compounds are described *supra*.

In one embodiment, the subject method further comprises formulating the identified compound for administration to a non-human animal or a human. Such formulations are described *supra*.

In another embodiment, the subject method further comprises producing or synthesizing the compound that is tested on the genetically modified animal. Such methods are described *supra*.

In an alternative embodiment, the invention provides a method of identifying a compound that reduces glucose uptake into liver, fat or muscle cells, such as, for example, in the treatment of obesity, said method comprising: (a) administering a compound that enhances glucose uptake to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal and determining the glucose uptake into liver, fat or muscle cells; (b) administering a compound to the animal and determining the glucose uptake into liver, fat or muscle cells of the animal, wherein a similar or reduced uptake at (b) compared to (a) indicates that the compound reduces glucose uptake into liver, fat or muscle cells.

Preferably, the animal is maintained on a diet comprising high glycemic index food, such as, for example, carrots or food supplemented with sucrose.

Preferably, the compound that enhances glucose uptake into liver, fat or muscle cells acts via a Cbl-mediated mechanism, which can be verified using the genetically modified Cbl-deficient animal, such as by determining the ability of the compound to enhance glucose uptake into liver, fat or muscle cells of Cbl-deficient mice according to a method described herein. In this case, step (a) *supra* of administering a compound that enhances glucose uptake into liver, fat or muscle cells comprises (i) administering a compound to a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (ii) determining the glucose uptake into liver, fat or muscle cells of the animal, wherein enhanced glucose uptake into liver, fat or muscle cells of the animal compared to a Cbl-deficient

animal to which the compound has not been administered indicates that the compound enhances glucose uptake into liver, fat or muscle cells.

In an alternative embodiment, the invention provides a method of identifying a compound that reduces glucose uptake into liver, fat or muscle cells comprising: (a) administering a compound to a non-human animal expressing a functional Cbl protein and determining the glucose uptake into liver, fat or muscle cells of the animal; (b) determining the glucose uptake into liver, fat or muscle cells of a genetically modified non-human animal comprising a genetic modification within an allele of its Cbl locus wherein said genetic modification reduces or prevents expression of functional Cbl in said animal; and (c) comparing the glucose uptake into liver, fat or muscle cells of the animals at (a) and (b) wherein a comparable uptake between (a) and (b) indicates that the compound reduces glucose uptake into liver, fat or muscle cells.

Preferably, the animals are maintained on similar or identical diets, more preferably, on diets comprising high glycemic index food.

The range of compounds contemplated herein include Cbl inhibitory compounds or antagonists of a biological function of Cbl. In one embodiment, the compound is selected from the group consisting of: a peptide, including a peptide derived from Cbl and capable of inhibiting, reducing or repressing a Cbl function, including binding to a protein selected from the group consisting of CAP, CrkII and C3G; a Cbl dominant-negative mutant; a non-Cbl peptide inhibitors of Cbl; an antibody or antibody fragment which binds to Cbl and inhibits a Cbl function; a small organic molecule, and nucleic acid, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide inhibitor, an antisense nucleic acid directed against Cbl-encoding mRNA, or an anti-Cbl ribozyme.

In one embodiment, the subject method further comprises formulating the identified compound for administration to a non-human animal or a human. Such formulations are described *supra*.

In another embodiment, the subject method further comprises producing or synthesizing the compound that is tested on the genetically modified animal. Such methods are described *supra*.

A further aspect of the present invention provides methods for determining a modulator of the activity, formation or stability of a protein complex selected from the group consisting of: (i) a Cbl-APS complex; (ii) a Cbl-CAP complex; (iii) a Cbl-CAP-flotillin complex; (iv) a Cbl- C3G complex; (v) a Cbl-CrkII complex; and (vi) a Cbl-C3G-CrkII complex.

In their general form, the methods of the present invention comprise determining the association or dissociation of the protein complex, or the structure of the complex, in the presence and absence of a candidate compound or a candidate antibody. In accordance with the embodiment described herein, a modified association, dissociation, or structure, of the protein complex in the presence of a candidate compound or a candidate antibody indicates that the candidate is a modulator of the protein complex.

The association, dissociation, or structure of the complex may be determined by direct means, such as, for example, by determining real time association or dissociation constants in the presence and absence of the candidate, or modified binding of an antibody that recognizes a conformational epitope of the complex. Biosensors used essentially as described herein above, in the presence or absence of the candidate compound or antibody, are particularly suited to such applications.

Alternatively, the association, dissociation, or structure of the complex may be determined by indirect means, such as, for example, using a protein recruitment system, n-hybrid screen, reverse n-hybrid screen, plate agar diffusion assay, ELISA, or other well known assay format for detecting protein-protein interactions. Such indirect means generally use a reporter system to detect formation or dissociation of the protein complex.

Standard solid-phase ELISA assay formats are particularly useful for identifying antagonists of the protein-protein interaction. In accordance with this embodiment, one

of the binding partners (e.g. APS or CAP or CrkII or C3G or a portion thereof) is immobilized on a solid matrix, such as, for example an array of polymeric pins or a glass support. Conveniently, the immobilized binding partner is a fusion polypeptide comprising Glutathione-S-transferase (GST; e.g. a CAP-GST fusion), wherein the GST moiety facilitates immobilization of the protein to the solid phase support. The second binding partner (e.g. Cbl) in solution is brought into physical relation with the immobilized protein to form a protein complex, which complex is detected using antibodies directed against the second binding partner. The antibodies are generally labelled with fluorescent molecules or conjugated to an enzyme (e.g. horseradish peroxidase), or alternatively, a second labelled antibody can be used that binds to the first antibody. Conveniently, the second binding partner is expressed as a fusion polypeptide with a FLAG or oligo-histidine peptide tag, or other suitable immunogenic peptide, wherein antibodies against the peptide tag are used to detect the binding partner. Alternatively, oligo-HIS tagged protein complexes can be detected by their binding to nickel-NTA resin (Qiagen), or FLAG-labeled protein complexes detected by their binding to FLAG M2 Affinity Gel (Kodak). It will be apparent to the skilled person that the assay format described herein is amenable to high throughput screening of samples, such as, for example, using a microarray of bound peptides or fusion proteins.

A two-hybrid assay is described in US Patent No. 6,316,223 to Payan *et al.*, incorporated herein by reference. The basic mechanism described by Payan *et al.* is similar to the yeast two hybrid system. In the two-hybrid system, the binding partners are expressed as two distinct fusion proteins in a mammalian host cell. In adapting the standard two-hybrid screen to the present purpose, a first fusion protein consists of a DNA binding domain which is fused to one of the binding partners, and a second fusion protein consists of a transcriptional activation domain fused to the other binding partner. The DNA binding domain binds to an operator sequence which controls expression of one or more reporter genes. The transcriptional activation domain is recruited to the promoter through the functional interaction between binding partners. Subsequently, the transcriptional activation domain interacts with the basal transcription machinery of the cell, thereby activating expression of the reporter gene(s), the expression of which can be determined. Candidate bioactive agents that modulate the protein-protein interaction between the binding partners are identified by

their ability to modulate transcription of the reporter gene(s) when incubated with the host cell. Antagonists will prevent or reduce reporter gene expression, while agonists will enhance reporter gene expression. In the case of small molecule modulators, these are added directly to the cell medium and reporter gene expression determined. On the other hand, peptide modulators are expressible from nucleic acid that is transfected into the host cell and reporter gene expression determined. In fact, whole peptide libraries can be screened in transfected cells.

Alternatively, reverse two hybrid screens, such as, for example, described by Vidal *et al., Proc. Natl Acad. Sci USA 93*, 10315-10320, 1996, may be employed to identify antagonist molecules. Reverse hybrid screens differ from froward screens *supra* in so far as they employ a counter-selectable reporter gene, such as for example, *CYH2* or *LYS2*, to select against the protein-protein interaction. Cell survival or growth is reduced or prevented in the presence of a non-toxic substrate of the counter-selectable reporter gene product, which is converted by said gene product to a toxic compound. Accordingly, cells in which the protein-protein interaction of the invention does not occur, such as in the presence of an antagonist of said interaction, survive in the presence of the substrate, because it will not be converted to the toxic product. For example, a portion/fragment of Cbl that binds to APS or CAP or CrkII or C3G is expressed as a DNA binding domain fusion, such as with the DNA binding domain of GAL4; and the portion of APS or CAP or CrkII or C3G that binds Cbl is expressed as an appropriate transcription activation domain fusion polypeptide (e.g. with the GAL4 transcription activation domain). The fusion polypeptides are expressed in yeast in operable connection with the *URA3* counter-selectable reporter gene, wherein expression of *URA3* requires a physical relation between the GAL4 DNA binding domain and transcriptional activation domain. This physical relation is achieved, for example, by placing reporter gene expression under the control of a promoter comprising nucleotide sequences to which GAL4 binds. Cells in which the reporter gene is expressed do not grow in the presence of uracil and 5-fluororotic acid (5-FOA), because the 5-FOA is converted to a toxic compound. Candidate peptide inhibitor(s) are expressed in libraries of such cells, wherein cells that grow in the presence of uracil and 5-FOA are retained for further analysis, such as, for example, analysis of the nucleic acid encoding the candidate peptide inhibitor(s). Small molecules that antagonize the interaction are determined by incubating the cells in the presence of

the small molecules and selecting cells that grow or survive of cells in the presence of uracil and 5-FOA.

Alternatively, a protein recruitment system, such as that described in U.S. Patent No. 5, 776, 689 to Karin *et al.*, is used. In a standard protein recruitment system, a protein-protein interaction is detected in a cell by the recruitment of an effector protein, which is not a transcription factor, to a specific cell compartment. Upon translocation of the effector protein to the cell compartment, the effector protein activates a reporter molecule present in that compartment, wherein activation of the reporter molecule is detectable, for example, by cell viability, indicating the presence of a protein-protein interaction.

More specifically, the components of a protein recruitment system include a first expressible nucleic acid encoding a first fusion protein comprising the effector protein and one of the binding partners (e.g. APS or CAP or CrkII or C3G or a portion thereof), and a second expressible nucleic acid molecule encoding a second fusion protein comprising a cell compartment localization domain and the other binding partner (e.g. Cbl or a portion thereof). A cell line or cell strain in which the activity of an endogenous effector protein is defective or absent (e.g. a yeast cell or other non-mammalian cell), is also required, so that, in the absence of the protein-protein interaction, the reporter molecule is not expressed.

A complex is formed between the fusion polypeptides as a consequence of the interaction between the binding partners, thereby directing translocation of the complex to the appropriate cell compartment mediated by the cell compartment localization domain (e.g. plasma membrane localization domain, nuclear localization domain, mitochondrial membrane localization domain, and the like), where the effector protein then activates the reporter molecule. Such a protein recruitment system can be practiced in essentially any type of cell, including, for example, mammalian, avian, insect and bacterial cells, and using various effector protein/reporter molecule systems.

For example, a yeast cell based assay is performed, in which the interaction between Cbl and one or more of its binding partners results in the recruitment of a guanine

nucleotide exchange factor (GEF or C3G) to the plasma membrane, wherein GEF or C3G activates a reporter molecule, such as Ras, thereby resulting in the survival of cells that otherwise would not survive under the particular cell culture conditions. Suitable cells for this purpose include, for example, *Saccharomyces cerevisiae* cdc25-2 cells, which grow at 36°C only when a functional GEF is expressed therein, Petitjean *et al., Genetics 124*, 797-806, 1990) Translocation of the GEF to the plasma membrane is facilitated by a plasma membrane localization domain. Activation of Ras is detected, for example, by measuring cyclic AMP levels in the cells using commercially available assay kits and/or reagents. To detect antagonists of the protein-protein interaction of the present invention, duplicate incubations are carried out in the presence and absence of a test compound, or in the presence or absence of expression of a candidate antagonist peptide in the cell. Reduced survival or growth of cells in the presence of a candidate compound or candidate peptide indicates that the peptide or compound is an antagonist of the interaction between Cbl and one or more of its binding partners.

A "reverse" protein recruitment system is also contemplated, wherein modified survival or modified growth of the cells is contingent on the disruption of the protein-protein interaction by the candidate compound or candidate peptide. For example, NIH 3T3 cells that constitutively express activated Ras in the presence of GEF can be used, wherein the absence of cell transformation is indicative of disruption of the protein complex by a candidate compound or peptide. In contrast, NIH 3T3 cells that constitutively express activated Ras in the presence of GEF have a transformed phenotype (Aronheim *et al., Cell. 78*, 949-961, 1994)

In yet another embodiment, small molecules are tested for their ability to dissociate the protein complex of the invention, by an adaptation of plate agar diffusion assay described by Vidal and Endoh, *TIBS 17*, 374-381, 1999, which is incorporated herein by reference.

A further embodiment of the invention provides a method for determining a modulator of an interaction between Cbl or a portion of Cbl and a polypeptide selected from the group consisting of APS, CAP, CrkII and C3G or a portion of said polypeptide, said method comprising:

(i) determining the level of a protein complex selected from the group consisting of: (i) a complex comprising Cbl and APS; (ii) a complex comprising Cbl and CAP; (iii) a complex comprising Cbl and CrkII; (iv) a complex comprising Cbl and C3G; (v) a complex comprising Cbl and CAP and flotillin; and (vi) a complex comprising Cbl and CrkII and C3G in the absence of a candidate compound or candidate antibody; and

(ii) determining the level of said protein complex in the presence of a candidate compound or in the presence of said candidate antibody

wherein a difference in the level of said protein complex at (i) and (ii) indicates that the candidate compound or candidate antibody is a modulator of said interaction.

This embodiment of the invention applies *mutatis mutandis* to the determination of protein complexes comprising a portion of any one or more of the protein binding partners (i.e. Cbl, APS, CAP, CrkII or C3G).

It will be understood by those skilled in the art that any one or more of the assay methods for antagonists as described herein above can be adapted for this purpose. This is because the level of the protein complex in the presence or absence of a candidate compound or antibody is related to antibody binding in the case of ELISAs, or to cell survival or growth, in the case of hybrid screens or protein recruitment assays. ELISA-based assay formats are particularly suitable for this purpose, because they are readily quantifiable, by calibrating the detection system against known amounts of a protein standard to which the antibody binds. Such quantitation is well known to the skilled person.

It is to be understood that the modulators can be antagonists or inhibitors of complex formation or stability, or alternatively, agonists or promoters of complex formation and stability.

The modulators identified using the methods described herein are useful for the therapeutic or prophylactic treatment of diseases associated with associated with Cbl function, such as, for example, modifying fat deposition or lean muscle mass, the ratio of body fat to muscle or metabolic rate, fatty acid synthesis, fatty acid oxidation, ACC phosphorylation, ACC activity, ATP synthesis or aberrant glucose uptake or feeding

behaviour. In one embodiment, the compounds are used to treat a condition selected from the group consisting of: hyperglycemia, hyperinsulinemia, obesity, adult-onset obesity, non-insulin-dependent diabetes mellitus, type II diabetes, glucose intolerance, and hypertrophy or hyperplasia of the islets of Langerhans. In another embodiment, the compounds are used for cosmetic purposes, such as, for example, by bodybuilders or persons wishing to modify their weight or body content of fat or muscle.

Accordingly, another aspect of the invention provides a method comprising administering an effective amount of a Cbl antagonist to an animal or human subject to inhibit or reduce the expression or activity of Cbl in the subject. Preferably, the subject is a subject in need of treatment, such as a subject suffering from a condition selected from the group consisting of: elevated glucose uptake, reduced appetite or dietary intake, hyperglycemia, hyperinsulinemia, enhanced fat deposition or obesity, adult-onset obesity, aberrant fatty acid synthesis and/or fatty acid oxidation, non-insulin-dependent diabetes mellitus, type II diabetes, glucose intolerance, and hypertrophy or hyperplasia of the islets of Langerhans.

In one embodiment, the invention also provides a method of treating a feeding disorder characterized by reduced dietary intake or suppressed appetite in a subject said method comprising administering to the subject an amount of a Cbl antagonist effective to enhance the appetite or dietary intake of the subject. The method of the invention is particularly suited to the treatment of anorexia or bulimia.

In a related embodiment, the invention also provides a method of treating a feeding disorder characterized by reduced dietary intake or suppressed appetite in a subject said method comprising administering to the subject an amount of a compound that reduces expression of functional Cbl effective to enhance the appetite or dietary intake of the subject.

Preferably, the compound or Cbl antagonist is a compound or antagonist identified using a screening method described herein.

To determine an appropriate dosage for treatment, data from the cell culture assays or animal studies are used, wherein a suitable dose is within a range of circulating concentrations that include the ED_{50} of the active compound with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any Cbl inhibitor or antagonist used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models (e.g. any one or more of the mouse models described *supra* having genetic obesity-diabetes syndromes, such as hyperglycemia, hyperinsulinemia, and obesity) to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test Cbl inhibitor which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma maybe measured, for example, by high performance liquid chromatography.

In an alternative embodiment, the present invention provides for the use of a Cbl antagonist in the preparation of a medicament for the treatment of a condition selected from the group consisting of: elevated glucose uptake, reduced appetite or dietary intake, hyperglycemia, hyperinsulinemia, enhanced fat deposition, enhanced fatty acid synthesis, reduced fatty acid oxidation, reduced ATP synthesis, reduced ACC phosphorylation, enhanced ACC activity or obesity, adult-onset obesity, non-insulin-dependent diabetes mellitus, type II diabetes, glucose intolerance, and hypertrophy or hyperplasia of the islets of Langerhans.

In a still further aspect, the present invention provides a method of modulating the level of phosphorylated acetyl-CoA carboxylase (ACC) and/or ACC activity in a cell or subject comprising modulating the activity or amount of a Cbl protein. As ACC phosphorylation and/or ACC activity is involved in the modulation of fatty acid synthesis/oxidation and ATP synthesis, this method clearly extends to a method of modulating fatty acid synthesis and/or fatty acid oxidation and/or ATP synthesis.

Methods of determining such a modulatory compound are described *supra*.

The range of modulatory compounds contemplated herein include Cbl inhibitory compounds or antagonists of a biological function of Cbl. In one embodiment, the compound is selected from the group consisting of: a peptide, including a peptide derived from Cbl and capable of inhibiting, reducing or repressing a Cbl function, including binding to a protein selected from the group consisting of CAP, CrkII and C3G; a Cbl dominant-negative mutant; a non-Cbl peptide inhibitors of Cbl; an antibody or antibody fragment which binds to Cbl and inhibits a Cbl function; a small organic molecule, and nucleic acid, including nucleic acid encoding said peptide derived from Cbl or said non-Cbl peptide inhibitor, an antisense nucleic acid directed against Cbl-encoding mRNA, or an anti-Cbl ribozyme.

In a particularly preferred embodiment, the method modulates ACC phosphorylation and/or ACC acticity without significantly modulating the expression of AMPK.

In one embodiment, a modulator of ACC phosphorylation and/or ACC activity is used to treat a subject in need. In accordance with this embodiment, a modulator is formulated as a pharmaceutical compound as described *supra*.

Such modulators of ACC phosphorylation are particularly useful for the treatment of disorders associated with aberrant fatty acid synthesis and/or aberrant fatty acid oxidation, such as, for example, obesity or neurodegenerative disease, or for cosmetic purposes, such as, for example, bodybuilding or weight loss.

The various embodiments of the invention described herein for identifying compounds that are administered to animals are also suitably performed using isolated cells that have been previously derived from the animal or readily available as isolated cells, the only requirement being that the cell possesses the required Cbl phenotype to perform the assay. In one embodiment, the isolated cells are skeletal muscle cells, cardiac muscle cells, fibroblasts or fat cells (adipocytes). In lieu of using wild-type or normal animals having no Cbl deficiency, 3T3-L1 adipocytes can be used. The various embodiments apply *mutatis mutandis* to the use of such isolated cells.

The present invention is further described with reference to the following examples and the accompanying drawings.

Example 1

Methods.

Animals were housed at the BTF facility of the Garvan Institute of Medical Research, Sydney, New South Wales, Australia. All procedures undertaken in these animals have been reviewed by the Ethics committee of the Garvan Institute. Animals were fed *ad libitum* with standard rodent chow and housed in 12 hour light/dark cycle. Body weight and food intake were monitored weekly for 12 weeks from weaning.

For determining glucose tolerance, animals were fasted overnight. Glucose (2 g/kg body weight) was administered into the intraperitoneal cavity following removal of a blood sample to test basal glucose concentration. Blood samples were taken at 15 min intervals for the following 90 min. Glucose assay was performed using the glucose oxidase method.

Fasting blood samples were also taken for measurement of circulating lipids and cytokines. Adipose depots, muscle, liver, brain were excised and weighed and frozen for subsequent biochemical analyses.

Temperature was measured using a Rectal probe (BAT-10, Physitemp) at the beginning and the end of the light cycle.

For determining glucose transport in muscle and adipose tissue, animals were fasted overnight and whole soleus muscle or gonadal white adipose tissue explants were incubated *in vitro* in the absence or presence of insulin in a Krebs Ringer buffer.

Glucose uptake was measured using the radiolabelled 2-deoxyglucose method..

For determining adipocyte size, the adipocytes were isolated using the collagenase method and incubated overnight in 2% osmium tetroxide at 37 C. Cells were mounted on a slide and images were acquired using bright field microscopy. Cell diameter was determine using Adobe Photoshop software.

Results

Figures 1 through 6 show that Cbl-deficient mice have significantly higher body weight and dietary intake, however reduced fat deposition and smaller adipocytes, compared to otherwise isogenic non-mutant animals of the same gender. The body temperature of Cbl-deficient animals is also enhanced. Surprisingly, and in contrast to what was expected, disruption of the Cbl gene lead to enhanced basal glucose uptake into both adipocytes and muscle cells. The results are also summarized in Table 1 below.

Table 1

Phenotype of Cbl-deficient mice

Parameter	Phenotype in c-Cbl$^{-/-}$ animals compared to wild type Cbl$^{+/+}$ animals
Body weight	No change or slight increase in males
Food intake	Increased
Fat mass	Reduced
Adipocyte size	Reduced
Glucose tolerance	Increased
Temperature	Increased
Glucose transport into fat	Increased
Glucose transport into muscle	Increased
Fasting blood glucose	No change

In two independent experiments, the average body temperature of Cbl-deficient males and females increased as indicated in Table 2 below:

Table 2

Enhanced body temperature in Cbl-deficient animals (Average °C ± SEM)

Experiment No.	Males		Females	
	Cbl$^{+/+}$	Cbl$^{-/-}$	Cbl$^{+/+}$	Cbl$^{-/-}$
1	37.11°C ± 0.26°C	38.93°C ± 0.22°C	37.60°C ± 0.30°C	38.06°C ± 0.15°C
2	37.25°C ± 0.16°C	38.17°C ± 0.14°C	37.62°C ± 0.09°C	37.31°C ± 0.12°C

EXAMPLE 2

Glucose transport in muscles isolated from c-CBL$^{-/-}$ mice

Overnight fasted mice (16-18 weeks old) were sacrificed by cervical dislocation and the soleus muscles (SOL) and extensor digitorum longus (EDL) muscles removed immediately for incubation *in vitro*. After excision, SOL and EDL were transferred to individual 25 ml flasks containing 2 ml of oxygenated medium placed in a shaking water bath at 30°C. All incubation media were prepared from a pre-gassed (95% O_2/5% CO_2) stock of Krebs-Henseleit Bicarbonate buffer (KHB) (118.5 mM NaCl, 24.7 mM $NaHCO_3$, 4.74 mM KCl, 1.18 mM $MgSO_3$, 1.18 mM KH_2PO_4 and 2.5 mM $CaCl_2$, pH 7.4) supplemented with 2mM pyrovate, 8 mM mannitol and 0.1% w/v bovine serum albumin (BSA). The gas phase in the flasks was maintained at 95% O_2/5% CO_2 throughout the experiment.

The muscles were allowed to recover for 10 min. after removal of the final muscle. A 2-deoxyglucose (2-DOG) uptake assay was performed with 16 muscles from 4 mice at a time. Muscles were placed in new media without insulin, or with 300 μU/ml (2.2 nM) insulin or with 10000 μU/ml (72 nM) insulin for 30 min. An insulin concentration of 300 μU/ml was selected, as this was the insulin level found to elicit half-maximal insulin-stimulated glucose uptake in SOL in a pilot experiment. An insulin concentration of 10000 μU/ml was used to ensure maximal insulin-stimulated glucose transport.

Following the 30 min. incubation period, the medium was changed to KHB containing 2 mM pyrovate, 8 mM mannitol, 0.1% w/v BSA, 1 mM 2-DOG, [1-^{14}C]-Mannitol and 2-[2-6 ^{3}H]-DOG (Amersham Pharmacia Biotech Inc., Little Chafton, U.K.) to a specific activity of 0.128 μCi/ml and 0.083 μCi/ml, respectively. When present, the insulin concentration was the same as during the previous 30 min. incubation period. Labeled 2-DOG diffuses into muscle cells through sarcolemmal glucose transporters and is trapped as 2-DOG-6-phosphate while diffusion of labeled mannitol across the plasma membrane is limited, making it suitable as an extracellular marker. After 16 min. exposure to isotopes, muscles were briefly washed in ice-cold KHB, blotted on paper, placed in Eppendorph tubes, and immediately frozen in liquid nitrogen. Muscles were stored at –80°C until processed.

Frozen muscles were weighed and transferred to fresh Eppendorf tubes. After addition of 250 µl of 1 N NaOH the muscles were incubated at 65°C with occasional vortexing until dissolved. Then 250 µl of 1 N HCl was added and samples were centrifuged at 17000 g for 3 min. Aliquots of 350 µl of the resultant supernatant supernatant, or an aliquot of a standard (5 µl of [1-^{14}C]-Mannitol and 2-[2-6 ^{3}H]-DOG in 10 ml Milli Q water), or an aliquot of media or background samples (Milli Q water) were transferred to β-scintillation vials. Then 4.5 ml of scintillation liquid (Ultima Gold XR, Perkin Elmer Life Sciences, Boston, MA, USA) was added to each vial and, after mixing, the tubes were counted for 3 min. each in a β-scintillation counter (Beckman LS6000 SC, Beckman Coulter, Inc. Fullerton, CA, U.S.A.).

As shown in Figure 7 the rate of 2-deoxyglucose uptake was significantly upregulated in SOL isolated from c-CBL$^{-/-}$ mice compared to c-CBL$^{+/+}$ controls, indicating an enhanced metabolic rate in c-CBL deficient mice. In the presence of 300µM/ml insulin (submax), the amount of 2-deoxyglucose uptake was significantly increased in both SOL and EDL isolated from c-CBL$^{-/-}$ mice compared to c-CBL$^{+/+}$ mice. When exposed to sufficient levels of insulin to ensure maximal insulin-stimulated glucose transport (ie. 1000µU/ml insulin) there was a significant increase in 2-deoxyglucose transport in SOL isolated from c-CBL$^{-/-}$ mice compared to c-CBL$^{+/+}$ mice.

These results indicate that c-CBL$^{-/-}$ mice have increased insulin-stimulated glucose uptake (approximately 30% increase) in isolated SOL and EDL muscles, thereby indicating that c-CBL$^{-/-}$ mice have increased peripheral insulin sensitivity.

EXAMPLE 3

Glucose transport in fat explants from c-CBL$^{-/-}$ mice

Animals were sacrificed as described in Example 2 and epididymal fat pads were excised and placed in 15 ml tubes containing Hepes Krebs Ringer Phosphate Buffer (HKRP) (12.5 mM HEPES/pH 7.4, 120 mM NaCl, 6 mM KCl, 1.2 mM Mg SO_4, 1 mM Ca Cl_2, 0.4 mM NaH_2PO_4, 0.6 mM Na_2 HPO_4) supplemented with 2 mM sodium pyruvate and 2% BSA. The tissue was then minced using scissors until pin-head size pieces were obtained. Approximately 50 µl of fat explants were placed in 24-well plate

wells containing 0.45 ml of HKRP buffer. Explants were incubated in the absence or presence of 0.05 nM or 1 nM insulin for 15 minutes at 37°C. Glucose transport was assayed using the 2-deoxyglucose method essentially as described in Example 2. The assay was initiated by the addition of 100 µM 2-Deoxy-[^{3}H] glucose (1.5 µCi/ml). Non-specific 2-Deoxy-[^{3}H] glucose uptake was determined in the presence of Cytochalasin B (50 µM). After 10 min, the assay was terminated by washing the cells rapidly three times with ice-cold phosphate-buffered saline (PBS). Fat explants in wells were collected and weighed. Explants were then placed in vials containing scintillation liquid and counted next morning as described above. ^{3}H cpm were normalised by tissue weight. Statistical analysis was performed using the Student's t test (Sigma Plot software).

As shown in Figure 8 the amount of 2-deoxyglucose incorporated into fat explants from c-CBL$^{-/-}$ (KO) mice was increased in the presence of both 0.05nM and 0.1nM of insulin compared to c-CBL$^{+/+}$ (WT) mice. These data indicate that c-CBL$^{-/-}$ mice have increased insulin-stimulated glucose uptake in fat explants compared to c-CBL$^{+/+}$ mice, indicating increased peripheral insulin sensitivity in knockout mice.

EXAMPLE 4

Measurement of resting metabolic rate of c-CBL$^{-/-}$ mice

Measurement of resting metabolic rate of mice was performed essentially as described in Withers, P. C. *Australian Journal of Zoology 49*:445-461, 2001. Briefly, open-circuit respirometry was used to measure the rates of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) at a controlled temperature of 25 (+/- 1°C).

Mice were removed from their nest box, weighed to ± 0.1 g and then placed into a metabolic chamber that consisted of a 500 ml glass jar sealed with a rubber stopper. Compressed air was passed through the chamber at 400 mL min^{-1}, controlled by a Brooks 5871-A mass-flow controller, to maintain levels of O_2 above 20% and CO_2 below 1%. Excurrent air passed though a column of drierite to remove water vapour, then through a Qubit S152 infrared CO_2 analyser then a Servomex 0A 184 paramagnetic O2 analyser. At the conclusion of the trial, the mouse was removed from

the chamber and its Tb was measured immediately using a plastic-sheathed thermocouple with a RS Components 611234 thermocouple meter. Baseline values of background O_2 and CO_2 were established for at least 15 min before and after each metabolic trial. Analog voltage outputs were recorded using Protek 506 (for CO_2) and Thurlby 1905a (for O_2) digital multimeters, and their RS232 outputs were recorded with a PC using a custom Visual Basic program. The metabolic system was calibrated using a butane flame (Withers 2001). Resting VO_2 and VCO_2 were determined using a custom Visual Basic program, using the formulae of Withers (2001).

As shown in Table 3 c-CBL$^{-/-}$ mice show increased oxygen consumption compared to c-CBL$^{+/+}$ mice, ie. c-CBL$^{-/-}$ mice show increased VO_2 and VCO_2.

The increased oxygen consumption observed in c-Cbl$^{-/-}$ animals indicate an elevation of resting metabolic rate. This result is consistent with lower plasma and tissue lipid levels observed in c-Cbl$^{-/-}$ mice compared to c-Cbl$^{+/+}$ animals (as described in Examples 2 and 3).

Table 3

Analysis of metabolic rate of cCBL$^{+/-}$ mice

	Tb (°C)	VO2 (ml/g/h)	VCO2 (ml/g/h)	RQ	conductance	
WT	36.7	3.5	2.96	0.85	0.272	**mean**
	0.318	0.120	0.121	0.05	0.009	**se**
	P<0.02	P<0.001	P<0.002	P<0.04	P<0.01	
c-CBL KO	37.6	4.6	3.7	0.8	0.36	**mean**
	0.2	0.22	0.2	0.022	0.02	**se**

se - standard error

EXAMPLE 5

Modulation of ACC activity and glucose metabolism in $Cbl^{-/-}$ mice

Quadriceps muscles from 6 c-$Cbl^{+/+}$ and c-$Cbl^{-/-}$ animals were isolated and snap frozen in liquid nitrogen. Frozen tissues were powdered using a dismembranator (micro-dismembranator II, B. Braun Biotech, Göttingen, Germany). In this device, the tissues to be powdered were placed together with a metal ball in a small spherical metal chamber. All assemblies were performed in a container with liquid nitrogen. The assembled chamber was then vigorously vibrated on the dismembranator until the tissues became a fine powder (45 sec.- 1 min.) and scraped into an Eppendorf tube. The powdered muscles were resuspended in HES buffer (20 mM HEPES, pH 7.4, 1 M EDTA, 250 mM sucrose, 1 mM sodium orthovanadate), supplemented with 1% SDS and proteinase inhibitors (10 μg/ml each of phenylmethylsulfonyl fluoride, aprotinin and leupeptin) with a pestle mixer. The samples were solubilised for 2 hours at room temperature. The homogenate was centrifuged at 13,000 rpm for 10 min. at room temperature (Eppendorf centrifuge 2417R) and the pellet discarded. Protein concentration in supernatants was measured using BCA method according to manufacturer's instructions (Pierce). Samples (50 μg of protein) were resolved by SDS-PAGE and transferred to PVDF membranes and immunoblotted using antibodies specific for Uncoupling Protein 3 (UCP3), cytochrome C, GLUT4, Insulin Receptor, AMPK and phosphorylated ACC.

As shown in Figure 9A levels of phosphorylated ACC in $Cbl^{-/-}$ mice were significantly increased above the levels detected in wild-type mice, suggesting that Cbl regulates ACC phosphorylation. Interestingly, the level of AMPK (the kinase thought to phosphorylate ACC) detected in $Cbl^{-/-}$ mice was not enhanced (Figure 9B).

As a result of ACC phosphorylation, and thereby inhibition, the expression of several proteins involved in glucose metabolism were induced, namely cytochrome C (Figure 9C), uncoupling protein 3 (UCP3, Figure 9D), insulin receptor (IR, Figure 9E) and glucose transporter 4 (GLUT4, Figure 9F)

EXAMPLE 6

Determining the effect of Cbl on AMPK activity

Protein derived from quadriceps muscles from c-Cbl $^{+/+}$ and c-Cbl$^{-/-}$ animals is prepared essentially as described in Example 5. Samples (50 μg of protein) were resolved by SDS-PAGE and transferred to PVDF membranes and immunoblotted using an antibody that specifically binds the active (phosphorylated) form of AMPK obtained from Cell Signalling (Beverly, MA, USA).

This assay determines the effect of Cbl deficiency on the activity of AMPK, the kinase that is thought to regulate ACC activity.

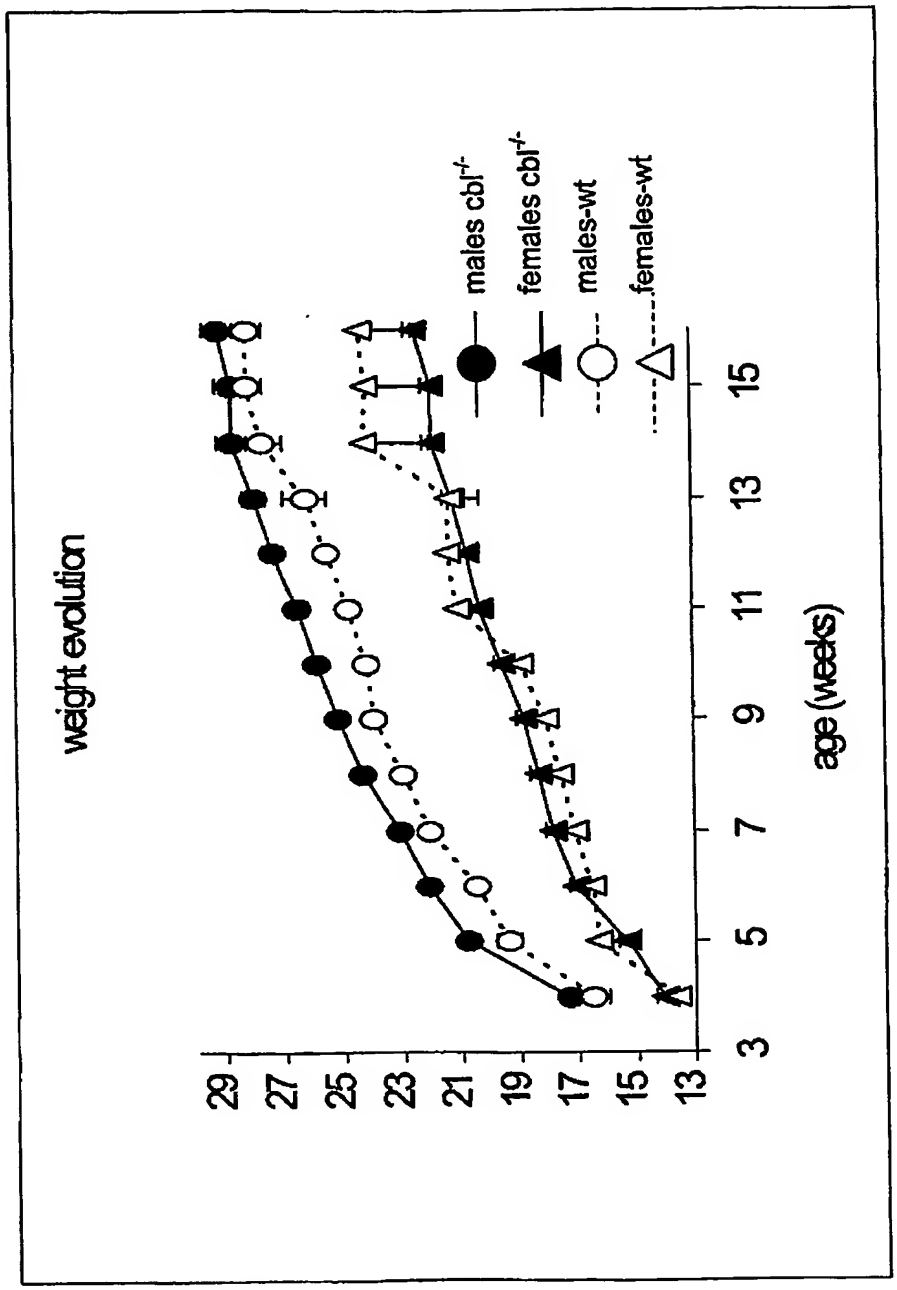

weight evolution
29
27
25
23
21
19
17
15
13
3
5
7
9
11
13
15
age (weeks)
males cbl-/-
females cbl-/-
males-wt
females-wt

FIGURE 1

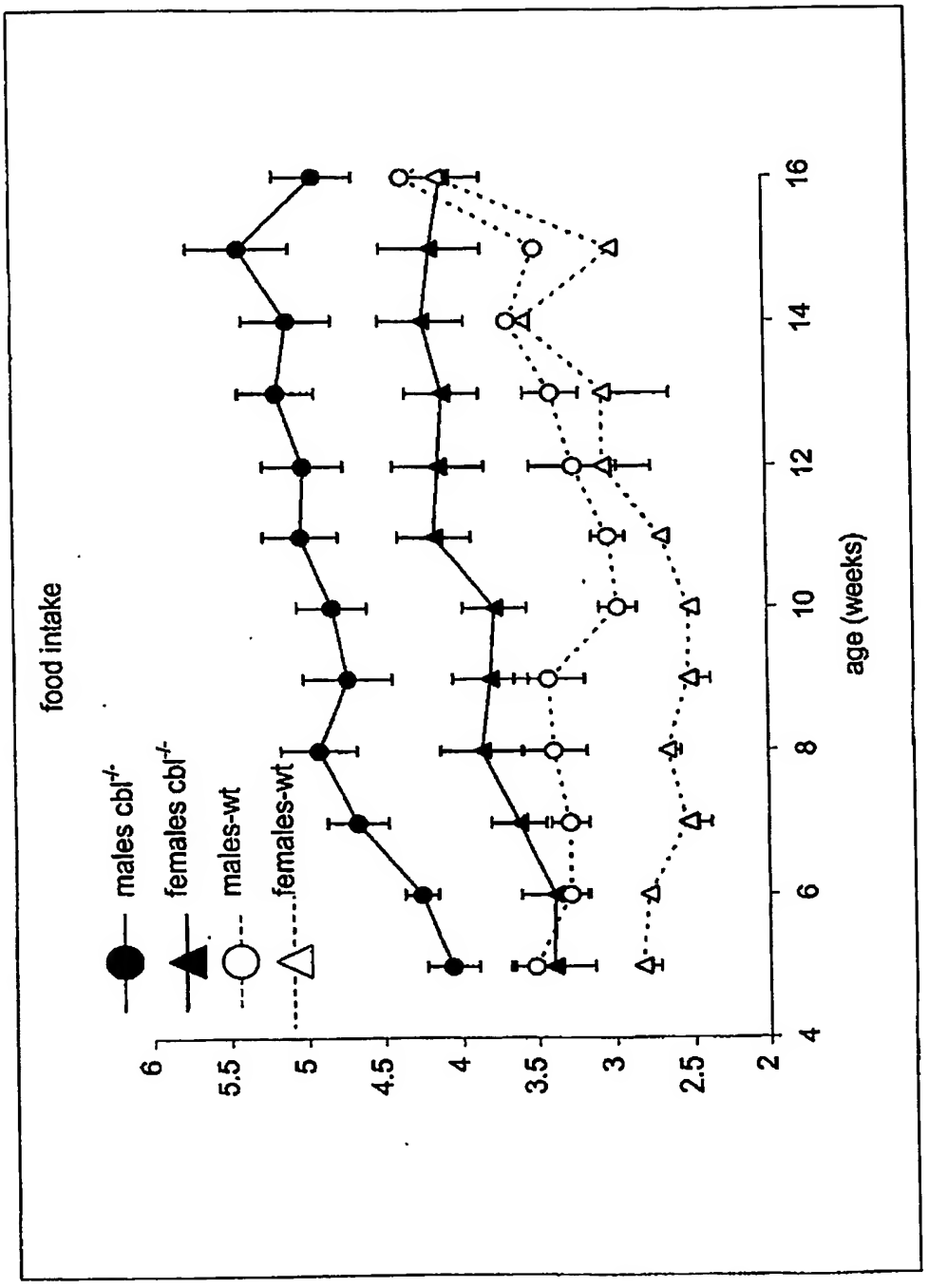

food intake
males cbl-/-
females cbl-/-
males-wt
females-wt
6
5.5
5
4.5
4
3.5
3
2.5
2
4
6
8
10
12
14
16
age (weeks)

FIGURE 2

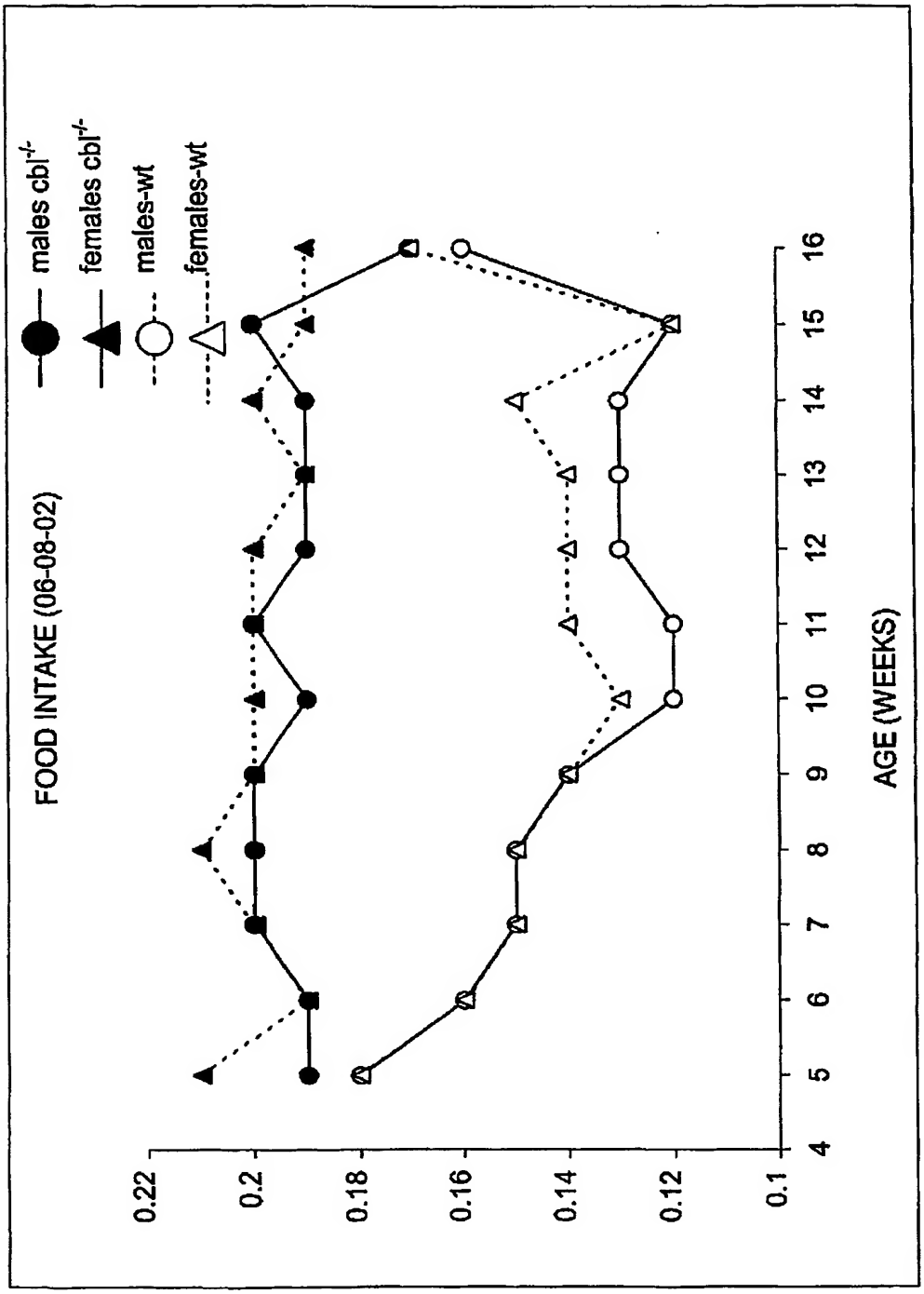

FOOD INTAKE (06-08-02)
males cbl-/-
females cbl-/-
males-wt
females-wt
0.22
0.2
0.18
0.16
0.14
0.12
0.1
4
5
6
7
8
9
10
11
12
13
14
15
16
AGE (WEEKS)

FIGURE 3

TISSUE WEIGHT

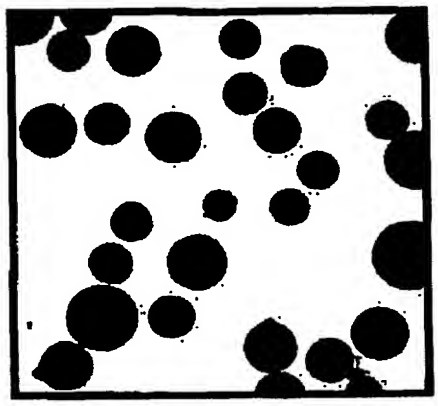
Wild Type

AVG	30.4	0.543	0.141	0.204		100	1.79	0.46	0.67
SEM	0.54	0.129	0.002	0.011			0.46	0.01	0.02
	BW	WAT	BAT	QUAD		BW	WAT	BAT	QUAD

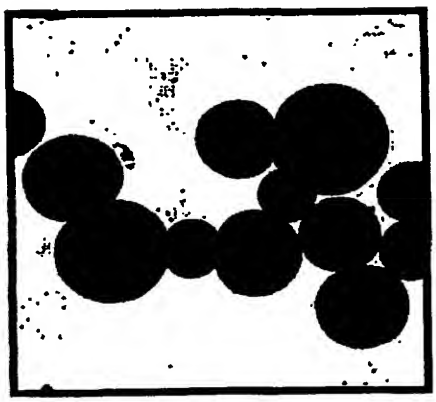
Cbl-deficient

AVG	30.00	0.226	0.073	0.206		100.00	0.75	0.24	0.69
SEM	0.95	0.021	0.005	0.013			0.07	0.01	0.04
	BW	WAT	BAT	QUAD		BW	WAT	BAT	QUAD

FIGURE 4



Cbl-deficient



Wild Type

FIGURE 5

ADIPOCYTE SIZE MALES

	diameter (μm)		VOLUME (pl/cell)		Lipid content (ng/cell)	
	AVG	SEM	AVG	SEM	AVG	SEM
WT	84.91	1.57	240.28	4.45	219.86	4.07
KO	49.44	0.90	82.43	1.25	75.42	1.14
KO 500	56.82	1.23	72	1.56	65.88	1.43

ADIPOCYTE SIZE FEMALES

	diameter (μm)		VOLUME (pl/cell)		Lipid content (ng/cell)	
	AVG	SEM	AVG	SEM	AVG	SEM
WT	80.48	2.32	204.6	5.9	187.3	5.4
KO	55.21	1.77	66.05	2.12	60.92	1.96

FIGURE 6

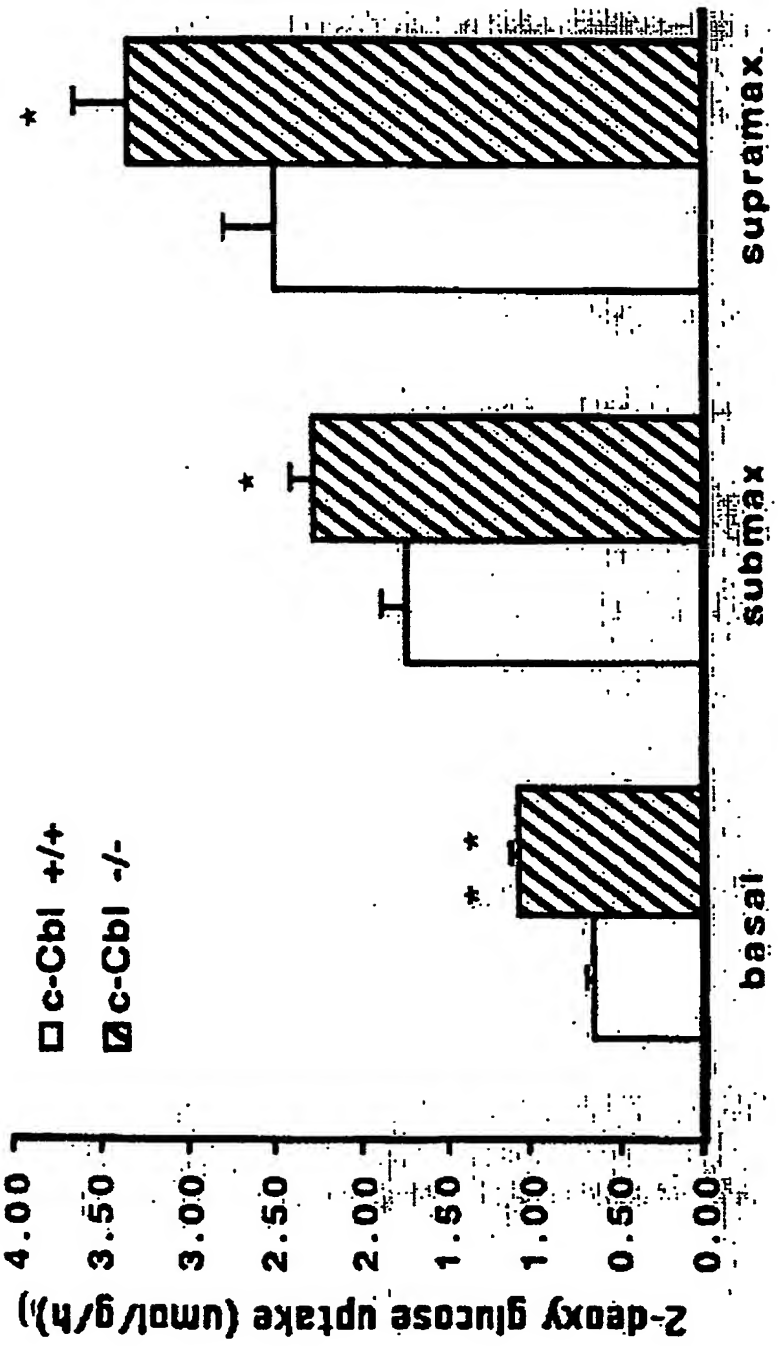

2-deoxy glucose uptake (umol/g/h))
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
□c-Cbl +/+
☒c-Cbl -/-
basal
submax
supramax
*
*
*
*

FIGURE 7A

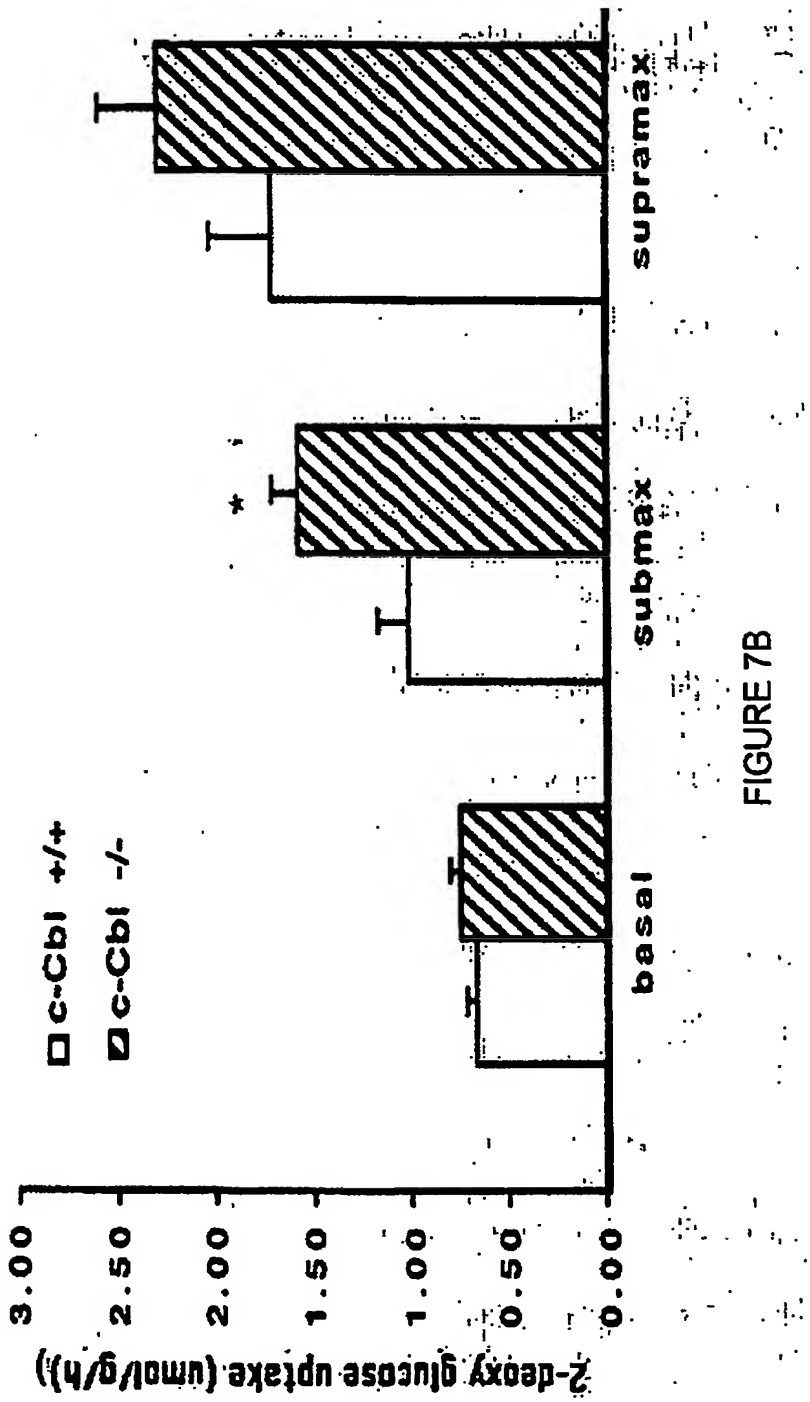

2-deoxy glucose uptake (umol/g/h))
3.00
2.50
2.00
1.50
1.00
0.50
0.00
basal
submax
supramax
*
c-Cbl +/+
c-Cbl -/-

FIGURE 7B

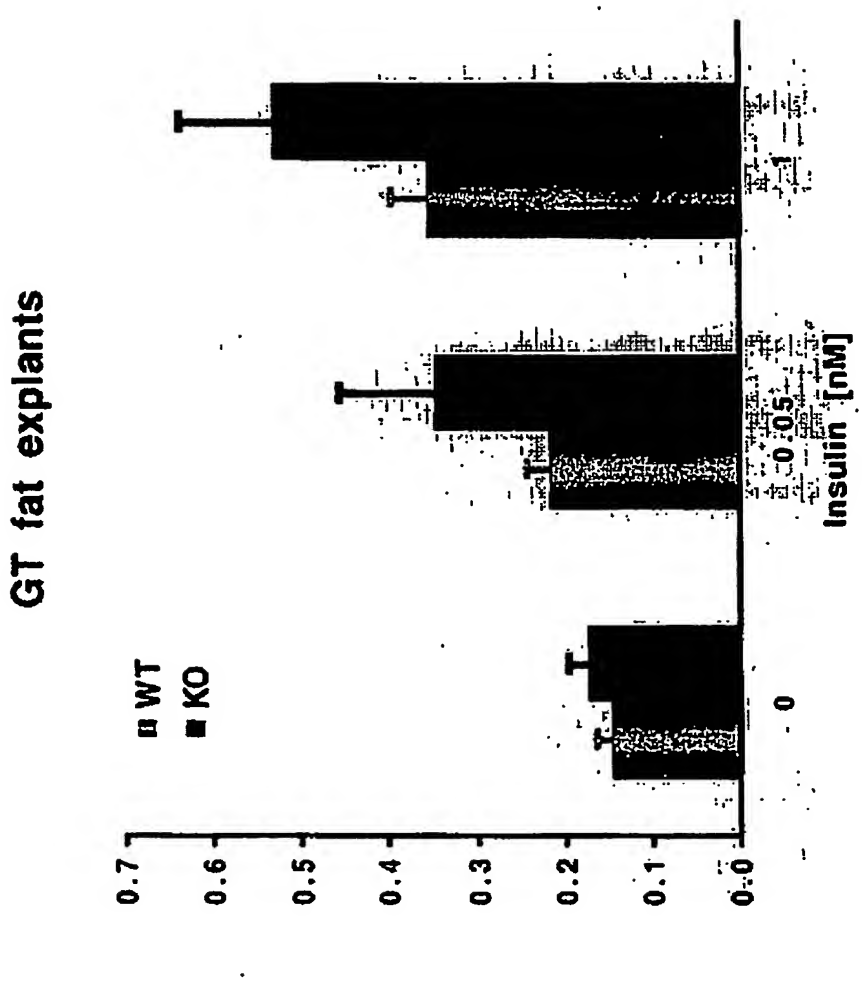


FIGURE 8

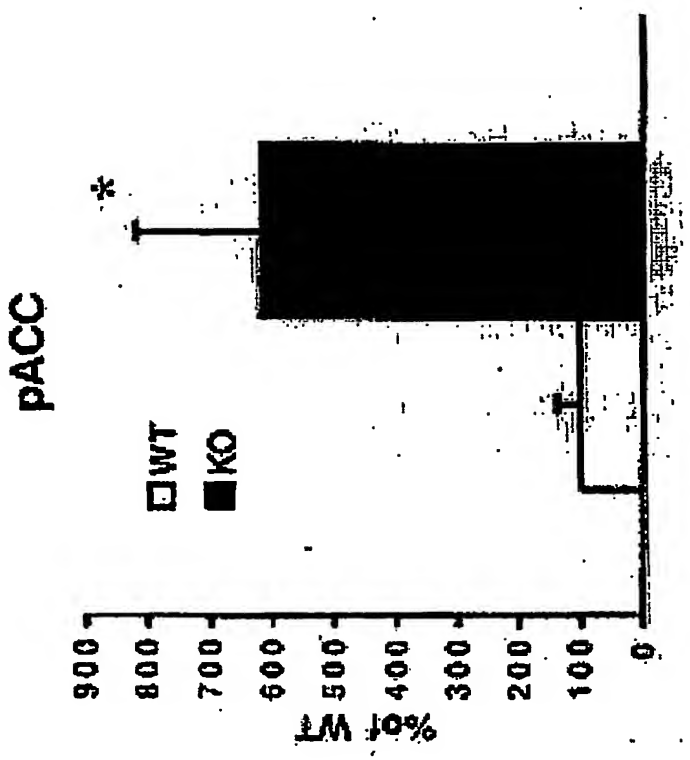


FIGURE 9A

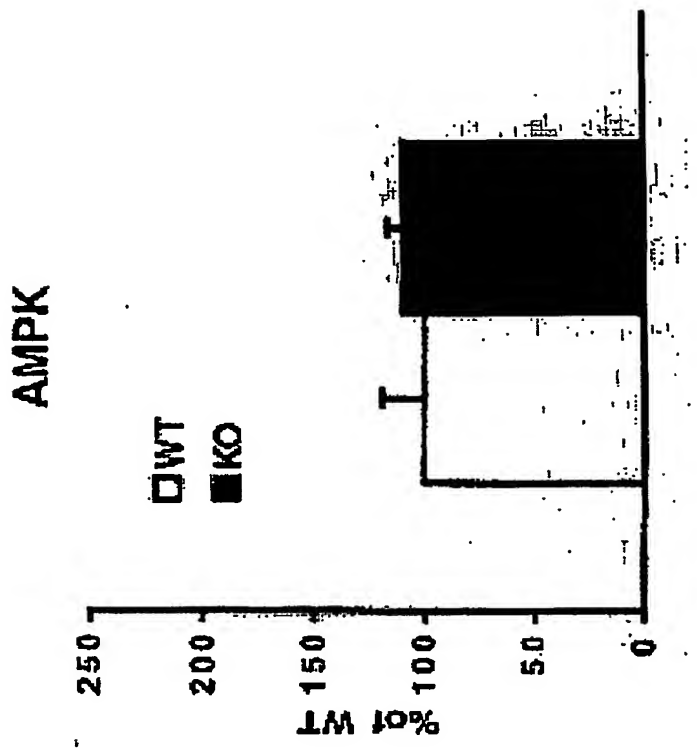


FIGURE 9B

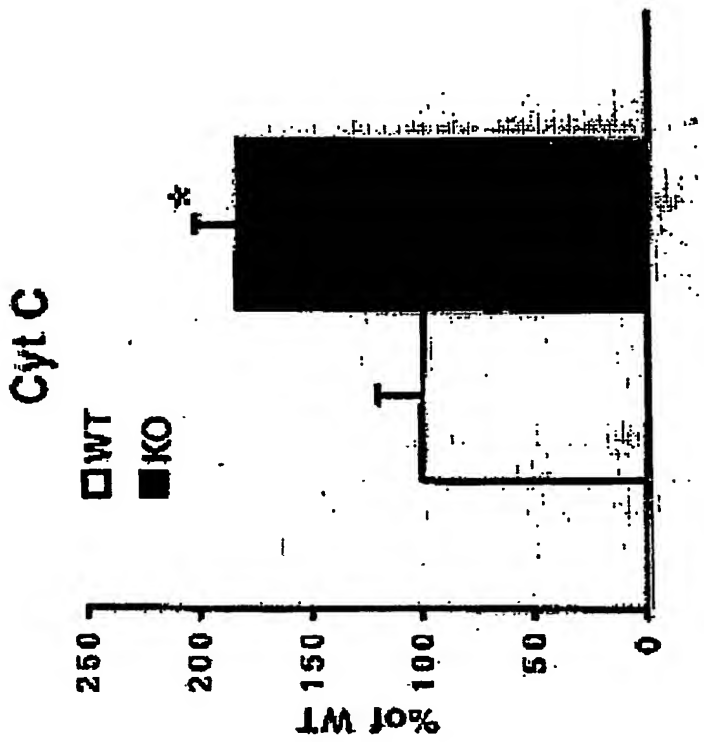


FIGURE 9C

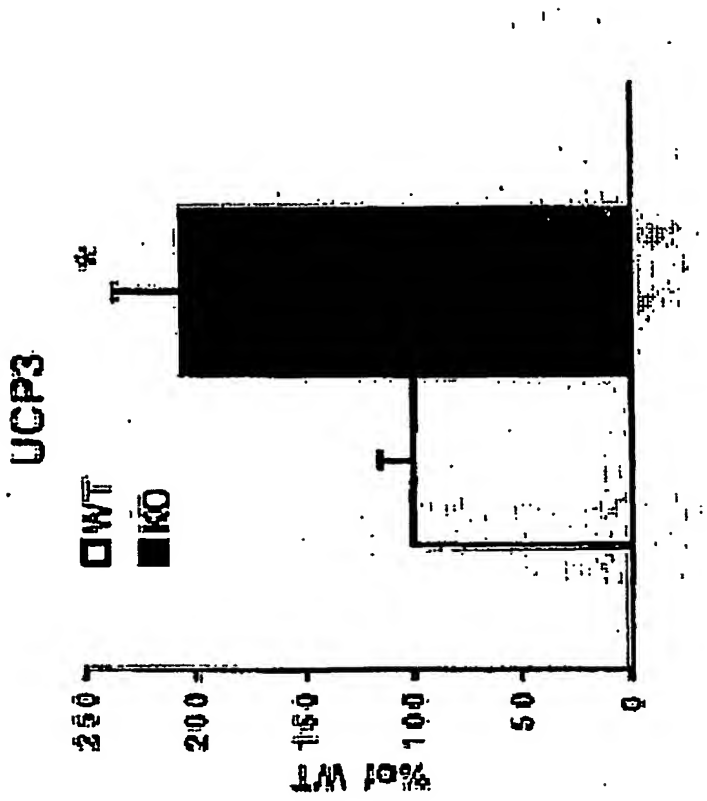


FIGURE 9D

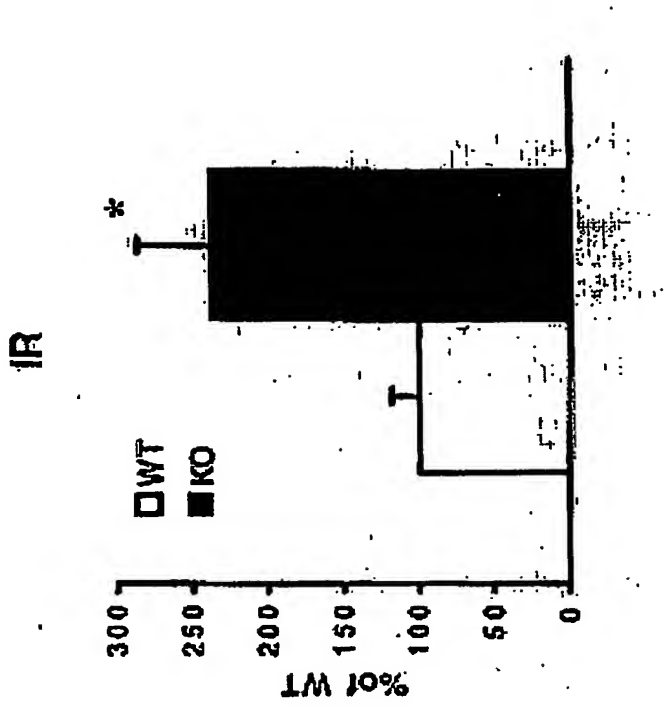


FIGURE 9E

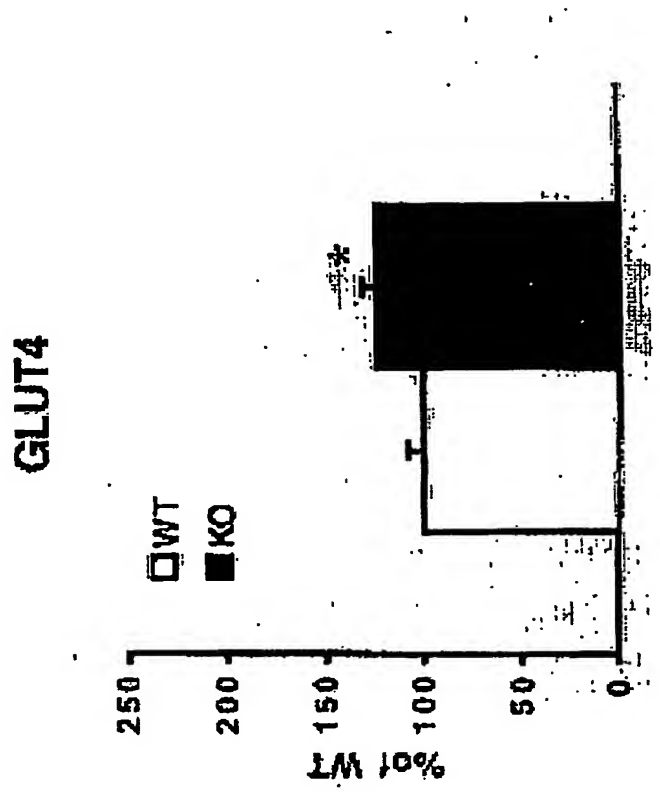


FIGURE 9F

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